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Total number of authors:
83

Published in:
Phytochemistry Reviews

Link to article, DOI:
10.1007/s11101-017-9532-2

Publication date:
2018

Document Version
Peer reviewed version

Link back to DTU Orbit

Citation (APA):
Dudnik, A., Almeida, A. F., Andrade, R., Avila, B., Bañados, P., Barbay, D., Bassard, J. E., Benkoulouche, M., Bott, M., Braga, A., Breitel, D., Brennan, R., Bulteau, L., Chanforan, C., Costa, I., Costa, R. S., Doostmohammadi, M., Faria, N., Feng, C., ... Forster, J. (2018). BacHBerry:: BACterial Hosts for production of Bioactive phenolics from bERRY fruits. *Phytochemistry Reviews*, *17*(2), 291-326. https://doi.org/10.1007/s11101-017-9532-2

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TITLE

BacHBerry: BACterial Hosts for production of Bioactive phenolics from bERRY fruits

RUNNING TITLE

BacHBerry

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Keywords: berries; bioprospecting; microbial cell factories; polyphenols; sustainable production.

Footnote

This article is written by the BacHBerry consortium (www.bachberry.eu) and represents the collective effort of all participating institutions. The authors are therefore listed in alphabetical order.

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Abstract

BACterial Hosts for production of Bioactive phenolics from bERRY fruits (BacHBerry) was a three-year project funded by the Seventh Framework Programme (FP7) of the European Union that ran between November 2013 and October 2016. The overall aim of the project was to establish a sustainable and economically-feasible strategy for the production of novel high-value phenolic compounds isolated from berry fruits using bacterial platforms. The project aimed at covering all stages of the discovery and pre-commercialization process, including berry collection, screening and characterization of their bioactive components, identification and functional characterization of the corresponding biosynthetic pathways, and construction of Gram-positive bacterial cell factories producing phenolic compounds. Further activities included optimization of polyphenol extraction methods from bacterial cultures, scale-up of production by fermentation up to pilot scale, as well as societal and economic analyses of the processes. This review article summarizes some of the key findings obtained throughout the duration of the project.

Introduction

BacHBerry (BACterial Hosts for production of Bioactive phenolics from bERRY fruits, www.bachberry.eu) has received funding from the European Commission's Seventh Framework Programme (FP7) under the theme KBBE.2013.3.1-01: "*Plant High Value Products - from discovery to final product*" with the goal of bringing together experts from the fields of plant biology, industrial biotechnology, analytical chemistry, and social sciences in order to assemble and validate a complete pipeline for discovery and microbial production of novel phenolic compounds of plant origin. The consortium consisted of twelve academic groups, five small and medium sized enterprises and one large enterprise from eleven different countries, namely Austria, Chile, China, Denmark, France, Germany, The Netherlands, Portugal, Russia, Switzerland and the United Kingdom. The diversity of partners has allowed access to a variety of expertise and to plant material that had not been characterized extensively.

Polyphenols as a source of bioactive molecules

Historically, plants have been not only important sources of nutrients and energy in the human diet but also have provided the fundamentals of traditional and modern medicine. The *Plantae* kingdom has been estimated to produce more than 200,000 different chemical compounds (Weckwerth 2003), including compounds with proven and potential medical applications. This is reflected in the high number of pharmaceutical products based on, or derived from, plant natural products, such as aspirin, morphine, quinine, paclitaxel and artemisinin (Cragg and Newman 2013). Among the different classes of specialized (i.e. secondary) metabolites phenylpropanoids stand out due to their diversity and ubiquity in the plant kingdom. Fleshy fruits, a rich source of bioactive components, such as ascorbate (vitamin C), terpenoids and polyphenols, are considered to have one the highest antioxidant capacity of commonly eaten foods (Halvorsen et al. 2006). Polyphenol content and composition can vary substantially between fleshy-fruit varieties (Carvalho et al. 2013; Miladinović et al. 2014; Kula et al. 2016), different cultivation conditions (Josuttis et al. 2013; Vagiri et al. 2013; Mazur et al. 2014), harvest times and as a result of different post-harvest factors (Kårlund et al. 2014; Stavang et al. 2015), but particularly between species (Moyer et al.

2001; McDougall et al. 2008; Ruiz et al. 2010; Marhuenda et al. 2016). Furthermore, plant polyphenols are increasingly being associated with putative bioactivities offering protection against several cardiovascular (Goszcz et al. 2015) and neurological diseases (Figueira et al. *in press*).

The hydroxystilbenes are a group of polyphenols with a C_6-C_2-C_6 skeleton with two or more hydroxyl groups, where a central carbon–carbon double bond is conjugated with two phenolic moieties (Quideau et al. 2011; Kasiotis et al. 2013). The most well-known representative of this group is resveratrol (*trans*-3,5,4'-hydroxystilbene) (**Figure 1a**). The compound has gained increasing attention with the discovery of the "French paradox", an observation that despite a diet with relatively high amounts of saturated fats, French people suffer a relatively low incidence of mortality from coronary heart disease (Renaud and de Lorgeril 1992; Catalgol et al. 2012). Like other polyphenols, stilbenes can be further decorated by *O*-methylation (e.g. resulting in 3-methoxy-resveratrol, or pinostilbene, and 3,5'-dimethoxy-resveratrol, or pterostilbene), acylation (hydrangenic acid) or by glycosylation (*e.g.* piceid and resveratroloside) (Kim et al. 2002; Becker et al. 2003; Zhang et al. 2009; Wang et al. 2015).

The flavonoids are one of the largest families of phenolic compounds. Flavonoids are characterized by their basic skeleton composed of three rings (Ghosh and Scheepens 2009), including two benzene rings (A & B) and one heterocyclic ring (C) (**Figure 1b**). So far, over 5,000 naturally occurring flavonoids have been characterized from various plants and they have been classified into six subgroups, including anthocyanidins (*e.g.* cyanidin and delphinidin); flavan-3-ols (*e.g.* catechin and epicatechin); flavonols (*e.g.* quercetin and kaempferol); flavones (*e.g.* luteonin and apigenin); isoflavones (*e.g.* genistein and daidzein), and flavanones (*e.g.* hesperetin and naringenin).

Within each subgroup, considerable variation can exist when it comes to phenolic composition of different fruits, and in particular of berries. Anthocyanin composition provides an excellent example of this diversity: the red-orange color of strawberries is due to the presence of pelargonidin-type anthocyanins in the flesh and skin whereas the deep purple-black color of blackcurrants is due to the accumulation of high amounts of delphinidin and cyanidin-type anthocyanins in the skin.

Anthocyanidins are flavonoids, which are characterized by a hydroxyl group in position 3 and a C-ring with a positively-charged oxonium ion (**Figure 1b**). Anthocyanins are water-soluble glycosides of anthocyanidins, in which sugars, consisting of one or more hexoses, are linked to the 3-hydroxyl group of the pyrylium ring (Heldt and Piechulla 2011). These compounds are responsible for the orange/red-to-blue coloration observed in some plants. The most common anthocyanidins include cyanidin (red), delphinidin (bluish-purple), malvinidin (reddish-purple), pelargonidin (orange-red), peonidin (pink) and petunidin (purple). The distribution of anthocyanidins can vary greatly among different berry species (**Table 1**).

Flavonoid compounds frequently are decorated with sugar moieties, sugar-acyl moieties (Giusti and Wrolstad 2003) and can be associated with other flavonoids. The patterns of decoration differ greatly amongst species (see Supplementary Tables S1 and S2).

Flavonoids are far more prevalent and diverse in berry species than in other common fruits and vegetables. High flavonol contents are observed in cranberry, lingonberry and blackcurrant (Häkkinen et al. 1999), anthocyanins are the most abundant polyphenol pigments (2-5 g kg^{-1} fresh weight) in berries (Määttä et al. 2001) and many simple phenolic acids are abundant in a wide range of berry species (Herrmann and Nagel 1989). Additionally, berries constitute one the most important dietary sources of ellagitannins such as sanguiin H6, lambertianin C (Törrönen 2009; Landete 2011) and condensed tannins such as the proanthocyanidins (Rasmussen et al. 2005; Hellström et al. 2009). Stilbenes, in contrast, are not that widespread in berries: resveratrol shows highest abundance in grapes (up to 20-30 mg kg^{-1} fresh weight), but small amounts of resveratrol, pterostilbene and piceatannol have been also detected in blueberry, cowberry, cranberry, deerberry and lingonberry (Jeandet et al. 1991; Paredes-López et al. 2010; Manganaris et al. 2014). It is clear that soft-fruit species contain a staggering diversity of distinct polyphenol derivatives whose potential is yet to be harnessed.

The market for polyphenolic compounds has seen substantial growth over the past few years, and is expected to exceed 850 million USD by 2018 (Aranaz et al. 2016). The major factors contributing to this trend are the growing consumer awareness regarding the benefits of polyphenol consumption and the increasing use of

polyphenol-containing extracts in food, beverage, and cosmetics products particularly in Asia (Jain and Ramawat 2013; Aranaz et al. 2016; Grand View Research Inc. 2016). The increase in demand also requires efficient and eco-friendly production processes, to improve on current manufacturing practices that mostly rely on extraction from various plant sources (*e.g.* roots, leaves or fruits) via complex downstream processing (Wang et al. 2016a). The BacHBerry consortium decided to address these challenges and set the following objectives: (i) to systematically analyze the phenolic contents in the large berry germplasm collections available from consortium members, (ii) to establish a publicly-available database of transcriptomic and metabolic data obtained from berry bioprospecting within, as well as outside of the project, (iii) to discover novel bioactivities in berry extracts against a range of human pathologies, such as Alzheimer's disease and Amyotrophic Lateral Sclerosis (ALS), by high-throughput screening with subsequent identification of functional biomolecules, (iv) to identify the corresponding biosynthetic genes and perform functional characterization of the respective gene products, (v) to assay a selection of the biosynthetic genes for functionality in Gram-positive bacterial hosts and use those to construct bacterial cell factories for phenolic production, (vi) to improve the production efficiency further by introducing modifications to the host metabolic networks predicted via rational design or computational tools developed within the project, (vii) to design and optimize cost-effective food-grade methods for extraction of phenolic compounds from bacterial fermentation broth, and (viii) to optimize fermentation conditions and subsequently upscale the production to pilot plant levels.

The project commenced with the selection, sampling and whole-metabolite profiling of berries. In order to explore the potential of the phytochemical diversity present in different berry species, we undertook an untargeted characterization of a wide collection of berries from different species/cultivars utilizing metabolomics-based methods to aid the selection of candidate berry extracts for bioactivity screening.

Phytochemical diversity in sampled berries

Although significant advances have been made in characterizing the polyphenolic complement of berries, particularly in the context of cultivated species and genotypes, there is limited literature available regarding

the phytochemical composition of wild and underutilized species/cultivars. Indeed, wild berries are commonly reported as particularly rich in diverse phenolic compounds, often as a result of phenolic-associated astringency having been bred out of cultivated species (Häkkinen et al. 1999). The phenolic diversity of individual berries has been highlighted in many publications (Määttä et al. 2003; Zadernowski et al. 2005; Milivojević et al. 2011), but studies that capture this broad diversity systematically are limited. In BacHBerry, we aimed not only to address this knowledge gap but also to go beyond the state-of-the-art by combining analyses of phenolic diversity with functional testing of berry extracts. The genera targeted in this study included *Rubus*, *Ribes*, *Vaccinium*, *Lonicera*, *Lycium*, *Aristotelia*, *Berberis* and *Ugni* collected from different locations in the world including Chile, China, Portugal, Russia and United Kingdom (see **Supplementary Table S3**).

Berry extract metabolite profiling

Fruit samples from a total of 112 species/genotypes were collected, extracted, and subsequently subjected to liquid-chromatography mass-spectrometry (LC-MS) metabolomic analysis (see details in Supplementary Materials S1). An untargeted method was used to generate a total of 1,890 mass spectral features (1,506 and 384 in positive and negative modes, respectively), which were integrated to generate the dataset used for statistical analysis. Principal component analysis (PCA) was used as a multivariate statistical analysis tool to identify those species, which had the most distinct phytochemical profiles (**Figures 2a and 2b**).

Using the four principal components it was possible to select the species/genotypes, which represented the broadest phenolic diversity (**Table 2**). In addition to selecting the outlier groups of species, it was also decided to include a small subset of samples (two *Rubus idaeus* genotypes), which did not separate from the majority of samples in the first principal components. This provided samples with phenolic profiles distinct from the outlier samples and standards for comparison between uncultivated/underutilized species and commonly grown species; in essence a commercial control. The fruits from the selected species (**Table 2**) were extracted and tested for bioactivity in yeast (*Saccharomyces cerevisiae*) models for pathological processes associated with several chronic disorders.

Berry extract bioactivity screening

Evidence for the protective role of polyphenols against chronic diseases has increased over the past 20 years (Figueira et al. *in press*; Goszcz et al. 2015). Neurodegenerative diseases (NDs) represent a group of chronic neurological conditions affecting millions of people worldwide, among which are the Alzheimer's Disease (AD), the Parkinson's Disease (PD), the Huntington's Disease (HD), and Amyotrophic Lateral Sclerosis (ALS). All these diseases have in common the aggregation and deposition of protein aggregates, namely of amyloid β42 (Aβ42) (O'Brien and Wong 2011), αSynuclenin (αSyn) (Shults 2006), huntingtin (HTT) (Miller-Fleming et al. 2008) and FUsed in Sarcoma (FUS) (Kwiatkowski et al. 2009; Vance et al. 2009), which represent the pathological hallmarks of AD, PD, HD and ALS, respectively. In addition, chronic activation of innate immune responses is a process closely associated with neurodegeneration. Its modulation is driven by persistent activation of key transcription factors, such as the Nuclear Factor of Activated T-cells (NFAT) and Nuclear Factor κB (NFκB), which upregulate pro-inflammatory responses creating a positive feedback loop further amplifying initial stimuli. It has been argued that disruption of this loop may represent an important strategy to mitigate the progression of NDs. The pleiotropic effects of polyphenols have been shown to interfere with aggregation-driven neurodegeneration as well as to attenuate chronic inflammatory processes, thereby improving health of cellular and animal models (Figueira et al, *in press*). Consequently, polyphenol-based therapies represent an underexplored strategy to minimize the huge social and economic impact of NDs.

The high degree of evolutionary conservation of fundamental biological processes among eukaryotes has established yeast as a validated model organism to decipher the intricacies of human pathologies, particularly NDs, to identify molecular targets amenable to therapeutic intervention as well as lead molecules with health-promoting potential (Kritzer et al. 2009; Su et al. 2010; Tardiff et al. 2012; Menezes et al. 2015). BacHBerry aimed at identifying phenolic bioactives from harnessing the diversity of phenolics in selected berry germplasm from cultivated, wild and underutilized species of berries. The yeast-based screening platform for bioactivity identification comprised a set of genetically modified *S. cerevisiae* strains expressing Green Fluorescent Protein (GFP) fused with Aβ42 (Bharadwaj et al. 2010), αSyn (Outeiro and Lindquist

2003), HTT (Krobitsch and Lindquist 2000) and FUS (Ju et al. 2011) under the control of a galactose-inducible promoter (**Figure 3a**). Upon activation of expression, these proteins start forming aggregates, which consequently has a negative impact on cellular growth and results in lower GFP fluorescence levels. In case an extract possesses bioactivity against one of the diseases, addition of the extract to the activated yeast cells reduces growth inhibition in the corresponding model. These yeast strains accelerated the identification of phenolic compounds with health-promoting attributes among the most chemically diverse samples identified by the metabolomic analysis (**Table 2**). For information on the procedures used during the screening see Supplementary Materials S1.

An illustrative example of how bioactivities for AD were identified is given in **Figure 4a**. Upon shift of cells to galactose medium to induce expression of GFP-Aβ42, yeast growth was impaired in comparison to the control strain indicating GFP-Aβ42 proteotoxicity. The treatment with polyphenol-enriched extracts of *Lycium chinense* significantly recovered growth of these cells revealing its protective role via modulation of Aβ42 toxicity. The bioactivities for PD, HD and ALS were screened using a similar approach, in cells expressing the respective disease proteins.

In addition, we have also used a yeast-based model for inflammatory signaling that is based on a Crz1 reporter-strain (Prescott et al. 2012; Garcia et al. 2016) (**Figure 3b**). Crz1 is a yeast orthologue of NFAT, which is an important modulator of inflammation in humans that is known to be involved in development of multiple disorders, such as the inflammatory bowel disease or the rheumatoid arthritis (Pan et al. 2013). Both Crz1 and NFAT are known to be activated by a serine/threonine protein phosphatase calcineurin (CaN) in a Ca^{2+}-dependent manner (Rusnak and Mertz 2000; Bodvard et al. 2013). The utilized reporter strain encodes the β-galactosidase gene (*lacZ*) under the control of a promoter bearing Crz1-binding sites, the Calcineurin-Dependent Responsive Element (CDRE), allowing the assessment of Crz1 activation through the measurement of β-galactosidase activity using chromogenic substrates (Garcia et al. 2016). Given the evolutionary conservation of NFAT and Crz1 activation mechanisms, in combination with the conserved activity of the immunosuppressant FK506 both in yeast and in humans, the yeast Ca^{2+}/CaN/Crz1 reporter assay represents a reliable tool to identify bioactives with potential to attenuate NFAT-mediated

inflammatory responses. The potential of *Lycium chinense* polyphenol-enriched extracts to attenuate inflammation is shown in **Figure 4b**, exemplifying the approach used in BacHBerry to filter for potential anti-inflammatory activities. The activation of Crz1 by $MnCl_2$, mimicking NFAT activation, led to high β-galactosidase activity and cell treatment with FK506 and *Lycium chinense* extract strongly reduced the β-galactosidase levels revealing its ability to modulate Crz1, and potentially NFAT, activation.

These examples illustrate the strategy used in the BacHBerry project to search for bioactive compounds interfering with pathological processes of NDs and inflammation. The yeast-based screening platform also included strains allowing the identification of metabolites with potential application for type II diabetes, hematological diseases and cancer (unpublished results).

Bioassay-guided fractionation compound discovery and candidate compound validation

This method of discovering new bioactive natural products ultimately depends on the availability of biological material and preparative- or semi-preparative-scale analytical methods with the capability of resolving mixtures of different classes of compounds typically present in berry extracts (Pauli et al. 2012). Semi-preparative chromatography was used to fractionate selected berry extracts with potential bioactive properties using a hybrid approach of bioassay-guided fractionation procedure (Yang et al. 2001; Weller and G. 2012; Tayarani-Najaran et al. 2013) and screening of pure compounds (Watts et al. 2010). Bioassay-guided fractionation typically involves the following steps: assessment of bioactivity, extraction of the biological material with different solvents and testing of bioactivity. Once bioactivity of an extract has been validated, the extract gets subjected to an iterative process of sub-fractionation/bioactivity testing until pure bioactive natural products are obtained for structural characterization. This approach benefits from exclusion of extracts that do not have bioactivity. However, this procedure requires extensive use of biological material and expensive materials and may result in the isolation of already-known natural products (Duarte et al. 2012). Another disadvantage is that the approach is based on the assumption that bioactivity is conferred by a pure compound, although this method can also be used for identification of bioactivities conferred by a cumulative interactions of several polyphenols. Alternatively, pure-compound screening relies on an initial

isolation and structural elucidation of the individual compounds present in the biological extract followed by bioassay screening. This strategy allows the researcher to focus solely on novel compounds, without re-discovering compounds with well-annotated bioactivity. However, it may also lead to the identification of novel compounds with no bioactivity (Duarte et al. 2012). The second method misses any synergistic interactions affecting bioactivities of berry phenolics.

The limited amount of biological material available restricted the number of iterations of fractionation and re-testing of fraction bioactivity possible for typical bioassay-guided fractionation approaches. As described previously, berry extracts typically comprise a relatively diverse and large pool of metabolites that surpass by far the throughput capability of the bioactivity assays used in this study. To overcome these challenges, a hybrid approach was adopted which consisted of several steps: (i) assessment of potential bioactivity present in extracts (as described above), (ii) fractionation of bioactive extracts (see Supplementary Materials S1), (iii) assessment of potential bioactivity present in fractions, (iv) mass-spectrometry-based chemical characterization of bioactive fractions, (v) bioactivity testing of pure candidate compounds (**Figure 5**). Although this approach shared some of the limitations of the other approaches, it did allow for the exclusion of non-bioactive biological extracts or fractions and focused on the identification novel compounds with potential bioactivities with limited requirement for biological material.

Following fractionation of the most promising berry extracts from the first round of screening using the yeast-based platform, isolated fractions were re-analyzed in order to detect bioactive fractions. Subsequently, metabolomic analyses were used for the identification of the individual compounds in each of the bioactive fractions. By utilizing this approach, a set of candidate compounds was defined and re-tested as pure compounds in the yeast-based platform, with further bioactivity validation in the mammalian cell models. Following the BacHBerry pipeline, this information then served as starting point for the engineering of the synthetic pathway for biosynthesis of one of the validated compounds in food-grade bacteria and its subsequent production in small-scale fermenters. The final step of the pipeline, namely re-confirmation of bioactivity of microbially-produced compounds extracted from fermentation broth in both yeast and and mammalian cell models, is currently underway.

Identification of metabolic pathways and regulators involved in phenolic production

The next step within the BacHBerry pipeline involved the identification of the biosynthetic genes and pathways corresponding to the newly discovered bioactives, in order to establish production of these compounds in bacterial hosts. In plants, the biosynthesis of polyphenols occurs via the phenylpropanoid pathway, where the aromatic amino acid $_{L}$-phenylalanine serves as a precursor. Depending on the enzyme combination, several compounds can be produced in bacteria, including cinnamic acid derivatives, lignin subunits, lignans, phenylpropenes and coumarins, all sharing a basic C_6-C_3 skeleton (Vogt 2010). Furthermore, the phenylpropanoid backbone can be extended with up to three acetyl-units derived from malonyl-CoA by chalcone synthase (CHS) or stilbene synthase (STS), polyketide synthases which generate various polyketides, such as flavonoids and stilbenes. The core pathways of phenolic biosynthesis are very well understood, with common enzymes, such as, phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), *p*-coumaroyl CoA ligase (4CL), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyltransferase (HCT), *p*-coumaroyl shikimate/quinate 3'-hydroxylase (C3'H), stilbene synthase and chalcone synthase, found in many plant species (**Figure 6**).

Phenolic chemodiversity in berries, as in other plants, derives from a variety of decorations to the backbone of phenolic structures that include hydroxylations, *O*-methylations, prenylations, aryl migrations, glycosylations, acylations and polymerizations. For example, anthocyanins are glycosylated on 3-*OH* positions of the C-ring (**Figure 1**) and many harbor additional glycosyl groups (for example 5'-*O*-glycosylation and 3'-*O*-glycosylation), methoxy groups (petunidin and malvinidin), or hydroxyl groups (which differentiate pelargonidin, cyanidin and delphinidin (**Figure 6 and Table 1**)). These decorations may affect the bioavailability, bioactivity and stability of polyphenolic compounds. It is therefore essential to develop a greater knowledge of the decorating enzymes specific to berry bioactives, such as those differentially decorating anthocyanins, flavonols, stilbenes, or condensed tannins. Many genes encoding enzymes involved in flavonol and anthocyanin decoration have already been characterized not only *in planta*, but also heterologously in microorganisms, such as *Escherichia coli* and *S. cerevisiae* (Tohge et al. 2005; Luo et al. 2007; Pandey et al. 2016; Wang et al. 2016a).

Berry bioactives identified within the project had special decorations, and those where the genes encoding the decorating activities had not been previously identified, required further research. In a recent work, the transcriptome of the commercial blueberry (*Vaccinium corymbosum*) was studied using RNA sequencing technology (RNA-seq) in order to discover putative genes related to antioxidant production using next-generation sequencing (NGS) (Li et al. 2012). Similarly, we aimed to identify decorating enzymes and regulators of the biosynthetic pathways of berry bioactives by obtaining transcriptome sequences of target species at different stages of fruit development. This information was used to construct a database of transcriptome sequences for a broad range of berry species, especially those identified as target species containing the promising bioactive molecules. In addition, the overall production of polyphenols is regulated by a range of transcription factors and these were also investigated within the BacHBerry project, with the objective of identifying markers for breeding enhanced levels of phenolic bioactives using the diverse germplasm in selected berry species.

Firstly, a library of reference sequences consisting of plant gene sequences encoding enzymes of the core phenolic biosynthetic pathways, which was subsequently used for BLAST searches of berry transcriptomes, was compiled. This library included well-known and experimentally validated anthocyanin, flavonol, stilbene and catechin biosynthetic enzymes from a wide range of plant species. Next, 13 germplasms spanning eight genera, seven families and seven orders were selected for RNA-seq of berry transcriptomes. Sequencing of selected berries at different stages of maturation was also conducted. The data have provided significant resources not only for understanding phenolic biosynthesis in berries, but also for investigating commonalities in genes expressed in berries (**Table 3**).

Multi-layer comparisons of the metabolomics and transcriptomics data of developmental stages, and targeted, as well as, non-targeted searches of candidate transcripts were performed to elucidate genes involved in specific phenolics biosynthetic pathways. Analyses of expression of these genes from different developmental stages of selected berry species were performed with the expVIP tool (Borrill et al. 2016).

These data were used to search for genes encoding putative decorating enzymes based on the list of molecules of potential interest as bioactives and having special decorations in target berry species. Candidates were identified mostly by searching for homologues of well-characterized plant genes. This was carried out with the help of MassBlast, a software developed within the BacHBerry project (https://github.com/averissimo/mass-blast; (Veríssimo et al. *submitted*)). We annotated all major enzyme families, including cytochromes P450 (CYP), 2-oxoglutarate-dependent dioxygenases (2-OGDs), UDP-glycosyltransferases (UGTs), BAHD acyltransferases, and *O*-methyltransferases (OMTs), in all 13 selected berry germplasms. Average counts per transcriptome are presented in **Table 4**. We also detected candidate transcript(s) for 60-80% of the known biochemical steps of the phenylpropanoid pathway, representing between 109-180 candidates per transcriptome. Approximately 40% of these candidates identified from the transcriptomes were full-length sequences. Furthermore, biochemical activities of some of enzymes encoded by the candidate genes were tested.

We also looked for transcripts encoding regulators of phenolic biosynthesis. Since the R2R3-MYB gene family operates extensively in controlling phenylpropanoid metabolism in plants (Stracke et al. 2001; Hartmann et al. 2005), initial searches were focused on identifying MYB-genes that were strongly expressed in berries. However, members of the bHLH and bZIP families of transcription factors were also identified in the berry RNA-seq databases since they may also play roles in regulating accumulation of phenolic bioactives (Hartmann et al. 2005). These data could be particularly useful in breeding programs for selection of plants with improved bioactive contents. The presence of genes encoding putative transporters of phenolic compounds was also examined. Excretion of target compounds produced by bacterial hosts into the culture medium via such transporters would simplify extraction procedures, and may also be necessary in case of any toxic effects of these molecules in bacteria. One transporter is currently being characterized functionally.

Gram-positive bacteria as cell factories for the efficient production of berry high-value phenolics

While the search for novel bioactives was under way, a set of well-characterized biosynthetic pathway was used for establishing a platform for production of phenolic compounds in Gram-positive bacteria. Two major

criteria were used for the selection of host organisms for this task: (1) they had to be robust, well-studied organisms with a long-standing history of industrial use and (2) the bacteria must have a generally regarded as safe (GRAS) status and/or be recognized as food-grade. Two industrially-important bacteria, namely *Lactococcus lactis* and *Corynebacterium glutamicum*, appeared as attractive candidates for the sustainable production of added-value food and pharmaceutical compounds (Lahtinen et al. 2011; Jojima et al. 2013). Both *L. lactis* and *C. glutamicum* are used worldwide in numerous industrial processes, which consequently led to significant amount of research work dedicated towards improving our knowledge of their physiology and genetics. These efforts have resulted in development of large toolboxes for (heterologous) gene expression and introduction of genetic modifications in these bacteria (Burkovski 2008; Pontes et al. 2011; Gaspar et al. 2013). Furthermore, high-throughput methodologies for transcriptome, proteome and metabolome analysis, and genome-scale metabolic models are available for these species (Krömer et al. 2004; Oliveira et al. 2005; Shinfuku et al. 2009; Gaspar et al. 2013). Combined with their general robustness, ease of handling and the GRAS status, both *L. lactis* and *C. glutamicum* have a strong potential for becoming platform organisms for production of polyphenolic compounds.

It should also be noted that during the earlier stages of the project, *E. coli* was also as a platform for reconstruction of polyphenol biosynthetic pathways. *E. coli* has a long history of use in research on microbial-based production of various value-added chemicals. Moreover, it is one of the best studied organisms, easy to handle, and there much larger set of genetic tools is available for this organism compared to other bacteria (Yu et al. 2011; Dobson et al. 2012; Chen et al. 2013). Furthermore, *E. coli* has been previously used for heterologous production of polyphenolic compounds (Pandey et al. 2016; Wang et al. 2016a).

Lactococcus lactis

L. lactis is a Gram-positive bacterium from the order of *Lactobacillales* that has been widely used in the food industry for the manufacturing of cheese, buttermilk and other dairy products. It is a low G+C aerotolerant bacterium with a relatively small genome and simple sugar metabolism: utilization occurs via glycolysis (Embden–Meyerhof–Parnas pathway) with lactic acid being the major end-product (Gaspar et al. 2013). Its

414 ability to ferment various carbohydrates, such as hexoses, pentoses and, in some strains, even complex
415 carbohydrates of plant origin, as well as its high alcohol, acid and stress tolerance makes this bacterium
416 suitable for the harsh environments commonly found under industrial production conditions. *L. lactis* strains
417 have been engineered to produce multiple commodity chemicals (*e.g.* 2,3-butanediol and butanol), as well as
418 food additives, including sweeteners (*e.g.* mannitol and xylitol), flavorings (*e.g.* acetaldehyde and diacetyl),
419 and vitamins. Furthermore, production of plant secondary metabolites, namely isoprenoids and stilbenes, has
420 been demonstrated in this organism (Neves et al. 2005; Katz et al. 2011; Gaspar et al. 2013; Song et al.
421 2014). In addition, *L. lactis* is well known as an excellent host for the expression of both prokaryotic and
422 eukaryotic proteins, and has been consequently used for development of live vaccine (Kunji et al. 2005;
423 Hernández et al. 2007; Frelet-Barrand et al. 2010; Pontes et al. 2011).

424 As depicted in **Figure 6**, in plants the biosynthesis of the core intermediate of the phenylpropanoid pathway,
425 *p*-coumaric acid, begins from L-phenylalanine and occurs in two steps: a deamination reaction catalyzed by
426 phenylalanine ammonia-lyase (PAL) and a hydroxylation reaction catalyzed by cinnamate 4-hydroxylase
427 (C4H). The latter enzyme belongs to the CYP family that is often challenging to express in active form in
428 prokaryotic systems (Dvora and Koffas 2013). However, in some bacteria and fungi there exists an
429 alternative pathway where L-tyrosine is converted directly into *p*-coumaric acid via the action of tyrosine
430 ammonia-lyase (TAL), hence bypassing the C4H step (**Figure 6**). As a first step towards establishing a
431 platform strain for the production of polyphenols in *L. lactis*, in collaboration with Nielsen and coworkers,
432 we identified several novel TAL enzymes that specifically produce high levels of *p*-coumaric acid from L-
433 tyrosine (Jendresen et al. 2015).

434 To test the potential of *L. lactis* to produce more complex phenolic compounds, we introduced a pathway for
435 the biosynthesis of resveratrol. The introduced pathway consisted of three enzymes: TAL, 4CL (4-
436 coumaroyl-CoA ligase, which converts *p*-coumaric acid to *p*-coumaroyl-CoA), and STS (stilbene synthase,
437 which catalyzes the condensation of *p*-coumaroyl-CoA and three molecules of malonyl-CoA to yield *trans*-
438 resveratrol, **Figure 6**). The corresponding genes were placed on the high copy number vector pNZ8048
439 under the control of the nisin-inducible promoter P_{nisA}. This promoter is a part of a tightly-controlled

inducible gene expression system which is based on the auto-regulatory mechanism controlling the production of the bacteriocin nisin (Kuipers et al. 1993; Kuipers et al. 1998). The producer *L. lactis* strain contains a chromosomally-integrated two-component system NisRK allowing induction of its target promoters by addition of sub-inhibitory concentrations of the bacteriocin nisin to the exponentially-growing cell culture (Linares et al. 2010). Upon cultivation of the producer strain in a chemically defined medium, production of *trans*-resveratrol was detected in the order of several mg l^{-1}. When TAL was replaced with a promiscuous PAL/TAL enzyme (MacDonald and D'Cunha 2007), additional production of small amounts of pinosylvin (*trans*-3,5-dihydroxystilbene) from $_{L}$-phenylalanine was observed. Furthermore, production of methylated variants of *trans*-resveratrol was also achieved (unpublished results). Obtained titers for *trans*-resveratrol are comparable to that obtained in an early work on *trans*-resveratrol production in both *E. coli* and yeast (Beekwilder et al. 2006; Choi et al. 2011). Current research efforts focus on improving the production efficiency through metabolic engineering of the central carbon metabolism for increased precursor supply.

Anthocyanins (glycosylated anthocyanidins) are another group of phenolic phytochemicals. In addition to their antioxidant properties, anthocyanins are valuable for their colors, which range from orange to blue (Falcone Ferreyra et al. 2012). They are of particular interest for the food industry as natural alternatives to replace synthetic dyes. Anthocyanins are synthesized from flavanones by flavanone 3-hydroxylase (F3H), which adds a hydroxyl group to the C3 position. Resulting dihydroflavonols are further processed by dihydroflavonol 4-reductase (DFR), which catalyzes reduction of the carbonyl group of the C4 position to form leucoanthocyanidins, which in turn are converted to anthocyanidins via the action of anthocyanidin synthase (ANS). Anthocyanidins are further stabilized through a C3-glycosylation reaction usually catalyzed by UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) to form the anthocyanidin 3-*O*-glucosides (Falcone Ferreyra et al. 2012) (**Figure 6**).

In order to reconstruct the anthocyanin production pathway in *L. lactis*, native genes and genes codon-optimized for *L. lactis* from various plant species coding for F3H, DFR, ANS and UFGT were placed under control of the nisin-inducible promoter P_{nisA} on a high copy-number vector or were integrated into the

chromosome of *L. lactis*. Each protein was taggedg with an N- or C- terminal strep-tag, and Western blots were performed to detect the heterologous proteins in cell-free extract of *L. lactis*. Activity of each enzyme was probed *in vitro* and *in vivo* and the reaction products were analyzed using high performance liquid chromatography (HPLC). Production of various types of cyanidin-derived anthocyanidins and anthocyanins by the engineered *L. lactis* strains was detected, further demonstrating the potential of this lactic acid bacterium as a production host for plant-derived bioactive compounds for food applications (unpublished results). To our knowledge, this is the first report of using lactic acid bacteria for the production of complex phenolic compounds.

Malonyl-CoA is a rate-limiting precursor in the biosynthesis of various phenolic compounds in bacteria (Xu et al. 2011; Bhan et al. 2013). Under physiological conditions, *L. lactis* cells use malonyl-CoA as a substrate in the chain elongation step of the fatty acid biosynthesis. FapR of *Bacillus subtilis* is a transcriptional repressor that directly senses the intracellular pool of malonyl-CoA. A metabolic biosensor based on the interaction of malonyl-CoA with FapR was previously designed in *Escherichia coli* (Xu et al. 2013). As a part of the efforts to enhance polyphenol production, a transcriptional biosensor for malonyl-CoA was constructed for the use in *C. glutamicum* but also proved to be fully functional in *L. lactis* and could therefore be used for screening of variants with an enhanced malonyl-CoA pool.

Among other activities that were pursued in the early stages of the project, we also tried to express plant CYPs in *L. lactis*, as many steps of the polyphenol biosynthetic pathway are catalyzed by these heme-bound monooxygenases. Functional expression of genes encoding membrane-anchored CYPs in prokaryotes is known to be notoriously difficult, and is limited by problems associated with the differences in intracellular localization (natively to the plant endoplasmatic reticulum), membrane composition, protein folding, and cofactor utilization. The latter element became especially crucial when attempting to express these proteins in *L. lactis*, because this, normally anaerobic, bacterium does not have the ability to synthetize heme, despite undergoing a metabolic shift to respiration when supplemented with an exogenous source of heme and being capable of regulating heme homeostasis (Pedersen et al. 2012).

We focused our efforts on CYP79A1 from *Sorghum bicolor* as it is a well-characterized CYP involved in dhurrin biosynthesis (Bak et al. 2000), and has been previously successfully produced in *E. coli* (Vazquez-Albacete et al. 2016). As a testbed for the expression of this CYP we have used the Waldo platform (Waldo et al. 1999) that was previously instrumental in producing functional CYP79A1 in *E. coli*. Briefly, the platform is based on a fusion of the membrane protein with a GFP folding reporter at the C terminus: the fused GFP is designed to act as a fluorescent reporter only when the membrane protein is properly folded and its C terminus is oriented towards the cytoplasm.

We detected CYP79A1-GFP by both fluorescence measurements of the *L. lactis* culture and in-fluorescence protein gels, but were not able to detect activity of the CYP in our *in vitro* tests. Purified membrane protein fractions from cell cultures grown both aerobically (with hemin supplementation) and anaerobically (with addition of hemin after purification of membrane proteins) did not show any product formation in our TLC-based assay (Blomstedt et al. 2012). These results suggested that CYP79A1 was probably misfolded in *L. lactis*, although we did not perform any follow-up experiments to confirm this hypothesis. The topic was not pursued any further because any CYP active in the biosynthetic pathways for the selected model compounds could be by-passed (for example the C4H-catalyzed step could be by-passed by using TAL instead of PAL).

Corynebacterium glutamicum

C. glutamicum is an important organism in industrial biotechnology for the microbial production of bulk chemicals, in particular of amino acids (Becker and Wittmann 2012). *C. glutamicum* is a very robust microorganism, which shows high resistance to the presence of small aromatic compounds (Liu et al. 2013), which renders *C. glutamicum* a very promising host for the production of pharmacologically interesting plant-derived polyphenols (Marienhagen and Bott 2013). Until recently, the activity of a complex catabolic network for aromatic compounds meant that *C. glutamicum* was not used for the production of aromatic compounds (except for aromatic amino acids) (Shen et al. 2012) due to rapid degradation of the products of interest. However, *C. glutamicum* can be easily modified genetically and a toolbox for the high-level expression of heterologous genes originating from other organisms, has become available, and is based on a

set of strong and inducible promoters (Eggeling and Bott 2005; Pátek and Nešvera 2013; Kortmann et al. 2015).

Surprisingly, in initial experiments, it turned out that *C. glutamicum* is able to grow on naturally occurring phenylpropanoids such as *p*-coumaric acid, ferulic acid, caffeic acid and 3-(4-hydroxyphenyl)-propionic acid as sole carbon and energy sources. The maximum growth rates on the selected phenylpropanoids ranged from 0.15 to 0.23 h^{-1} corresponding to doubling times of 3 to 4.5 hours (Kallscheuer et al. 2016a). We suspected that the activity of this pathway would come into conflict with any attempt to produce polyphenols as both pathways compete for phenylpropanoids. Unfortunately, the underlying metabolic pathway responsible for the degradation of phenylpropanoids in *C. glutamicum* was unknown at the start of the BacHBerry project. Global gene expression analyses were conducted, which led to the identification of a gene cluster consisting of seven genes (designated *phdT, phdA, phdR, phdB, phdC, phdD* and *phdE)*. In response to feeding of the above-mentioned phenylpropanoids these genes were upregulated up to 100-fold (Kallscheuer et al. 2016a). In subsequent studies, it turned out that the genes in this cluster code for the enzymes of the phenylpropanoid catabolic pathway in *C. glutamicum*. The gene *phdT* codes for a phenylpropanoid transporter protein, whereas *phdA* and the predicted operon *phdBCDE* code for enzymes involved in the degradation of phenylpropanoids. The *phd* gene cluster is transcriptionally controlled by a MarR-type repressor encoded by *phdR*. Cultivation experiments conducted with *C. glutamicum* strains carrying single-gene deletions showed that loss of *phdA, phdB, phdC* or *phdE* abolished growth of *C. glutamicum* with all tested phenylpropanoid substrates. The intracellular accumulation of pathway intermediates determined via LC-ESI-MS/MS in single-gene deletion mutants showed that the *phd* gene cluster encodes for enzymes involved in a CoA-dependent, β-oxidative deacetylation pathway, which is essential for the utilization of phenylpropanoids in *C. glutamicum*. The identified pathway thereby represents a peripheral catabolic route responsible for chain shortening of phenylpropanoids to benzoic acids and acetyl-CoA (**Figure 7**). *C. glutamicum* converts the resulting intermediates benzoate, 4-hydroxybenzoate and protocatechuate to succinyl-CoA and acetyl-CoA by central degradation pathways for aromatic compounds (especially the well-characterized β-ketoadipate pathway), and catabolites are ultimately channeled into the

tricarboxylic acid cycle (Shen and Liu 2005) (**Figure 7**). Unexpectedly, *C. glutamicum* failed to grow on the phenylpropanoid, cinnamic acid, which lacks ring *para*-hydroxylation. Astonishingly, a *C. glutamicum* strain with a single deletion of the gene *phdR* coding for the identified transcriptional repressor was able to grow with cinnamic acid as sole carbon and energy source (Kallscheuer et al. 2016a). This indicated that PhdR has a pronounced specificity for hydroxylated phenylpropanoids, especially when hydroxylated in *para*-position of the aromatic ring. In the course of our studies, cinnamic acid was the only phenylpropanoid tested lacking this hydroxylation, so the complete specificity of this catabolic pathway of phenolics in *C. glutamicum* remains unknown.

During construction of the *C. glutamicum* platform strain for plant polyphenol production, four gene clusters, comprising 21 genes, including four genes of the *phd* cluster involved in the catabolism of aromatic compounds, were deleted. The resulting platform strain *C. glutamicum* DelAro^4 was unable to degrade any phenylpropanoid tested, and also could not utilize simple benzoic acids such as protocatechuate (Kallscheuer et al. 2016c). This strain was used as a chassis for the microbial production of polyphenols in particular, stilbenes and (2*S*)-flavanones, in *C. glutamicum*.

Plasmid-borne expression of codon-optimized genes coding for a 4CL from parsley (*Petroselinum crispum*), a chalcone synthase (CHS) and a chalcone isomerase (CHI) from *Petunia x hybrida* from the strong T7 promoter in this strain background enabled production of (2*S*)-flavanones (**Figure 7**). Maximal titers of 35 mg l^{-1} naringenin and 37 mg l^{-1} eriodictyol were obtained from the supplemented phenylpropanoids *p*-coumaric acid and caffeic acid, respectively. Expression of the *4cl* gene in combination with a codon-optimized gene coding for an STS from peanut (*Arachis hypogaea*) allowed for the production of the stilbenes pinosylvin, *trans*-resveratrol and piceatannol starting from supplemented phenylpropanoids cinnamic acid, *p*-coumaric acid and caffeic acid, respectively (Kallscheuer et al. 2016c) (**Figure 7**). Stilbene concentrations of up to 158 mg l^{-1} could be achieved in defined CGXII medium with 4% glucose and supplemented with cerulenin, a fatty-acid biosynthesis inhibitor that is known to enhance polyphenol production in bacteria by increasing the intracellular concentrations of malonyl-CoA (Santos et al. 2011; van Summeren-Wesenhagen and Marienhagen 2015; Cao et al. 2016). Engineering amino acid metabolism for an

optimal connection to the synthetic plant polyphenol pathways was conducted to enable stilbene production directly from glucose. Indeed, the additional heterologous expression of *aroH* from *E. coli* (coding for the first enzyme of the shikimate pathway responsible for the synthesis of aromatic amino acids) together with a codon-optimized gene coding for a TAL from *Flavobacterium johnsoniae* led to the production of up to 60 mg l^{-1} *trans*-resveratrol in the absence of supplemented *p*-coumaric acid (Kallscheuer et al. 2016c). Furthermore, *C. glutamicum* was used to produce *trans*-resveratrol from a cheaper precursor 4-hydroxybenzoic acid (HBA) through reversal of a microbial β-oxidative phenylpropanoid degradation pathway (Kallscheuer et al. 2016b). This novel synthetic pathway circumvents any need for the endogenous supply of aromatic amino acids as polyphenol precursors and in *C. glutamicum* instantly yielded 5 mg l^{-1} *trans*-resveratrol from supplemented HBA without any further optimization. Very recently, we also engineered *C. glutamicum* to produce more complex polyphenols including flavonols (kaempferol and quercetin) and *O*-methylated stilbenes (pinostilbene and pterostilbene). The obtained pterostilbene titer was 42 mg l^{-1}, which is comparable to the titer of 50 mg l^{-1}obtained in engineered *E. coli*. In *C. glutamicum*, for the flavonols kaempferol and quercetin titers of 23 mg l^{-1} and 10 mg l^{-1}, respectively, were detected. The aforementioned titers were obtained from the phenylpropanoids *p*-coumaric acid and caffeic acid , respectively, as precursors and represent the highest flavonol titers obtained in engineered microorganism until today (Kallscheuer et al. 2017).

The main bottlenecks for the production of polyphenols turned out to be the TAL activity as well as the intracellular availability of malonyl-CoA, representing the cosubstrate in the STS- and CHS-reactions. On-going efforts to improve polyphenol production in *C. glutamicum* therefore focus mainly on improving the activity of the rate-limiting enzymes and increasing the intracellular malonyl-CoA pool.

Other activities

As a part of this work, we also aimed at reconstruction of a previously unknown biosynthetic pathway for the flavonol fisetin (3,7,3′,4′-tetrahydroxyflavone, **Figure 8**). Fisetin is a potent anti-inflammatory, anti-cancer, and anti-oxidant compound (Suh et al. 2008; Funakoshi-Tago et al. 2011; Jash and Mondal 2014). Moreover,

it has been demonstrated to have a neuroprotective effect and also to limit the complications of type I diabetes (Maher 2009; Maher et al. 2011). Based on the molecular structure of this compound, we hypothesized that its biosynthesis should follow the general pathway of flavonol biosynthesis (**Figures 6 and 8**): a flavanone precursor would be converted into dihydroflavonol by F3H, and then further converted into the corresponding flavonol via the action of flavonol synthase (FLS). In the last step of the biosynthesis, a hydroxyl group would be added at the C3' position by flavonoid 3'-hydroxylase (F3'H). This part of the work was carried out in *E. coli*. Isoliquiritigenin (2',4',4-trihydroxychalcone) appeared to be the most suitable precursor, and when supplied to an *E. coli* culture expressing F3H and FLS from *Arabidopsis thaliana*, production of garbanzol and resokaempferol was detected. Resokaempferol could be further converted into fisetin by expression of F3'H genes from *A. thaliana* and *Pn. hybrida* fused with a cytochrome-P450 reductase (CPR) from *Catharanthus roseus* (Stahlhut et al. 2015). The three enzymes were then combined with the isoliquiritigenin biosynthetic pathway, consisting of TAL from *Rhodobacter sphaeroides*, 4CL from *Pr. crispum*, CHI from *Medicago sativa,* and a fusion protein of CHS from *Pn. hybrida* and CHR from *Astragalus mongholicus*, to yield 0.3 mg l^{-1} of fisetin (Stahlhut et al. 2015). This work allowed reconstruction of a complete pathway for the biosynthesis of fisetin from L-tyrosine as the starting precursor using enzymes from various sources, and the identified pathway is currently being transferred to Gram-positive hosts.

Bioinformatics and modelling

There were multiple activities within BacHBerry, both within the discovery and the microorganism engineering sectors, which required the use of bioinformatics tools. Hence, the mathematical modeling and computational parts of the project aimed at addressing three major problems. The first two were related to gene expression, either for performing functional genomics analysis using the RNA-seq data from berries or to design sequences for heterologous expression of plant genes in microbes. The third problem concerned metabolic network optimization for both a single population and for a community of microbes. We detail below the main results obtained on each of the above activities. Other subjects that were addressed as a part of this project were metabolic communication between the different species in a community (Milreu et al. 2014; Andrade et al. 2016) and modeling dynamic metabolic networks for potential application in strain

optimization (Costa et al. 2014; Hartmann et al. 2015a; Hartmann et al. 2015b; Costa et al. 2016; Hartmann et al. 2016).

Sequencing data analysis and plant functional genomics

Understanding which genes are differentially expressed during phenolic production by plants requires the capacity to undertake a rapid analysis of RNA-seq data. One of the main problems to be addressed in this case is the search for coding sequences that are homologous to those already present in sequence databases, which involves identification, selection and annotation of the best matches. Currently, such analyses rely heavily on manual curation as well as on the manual assembly of results obtained using different computational procedures and sequence databases. Automated processes become crucial in the light of the ever-increasing amounts of generated transcriptome data. Furthermore, automation of processes also allows improvement of the quality of the analyses, as the likelihood of human error grows with the amount of manual processing and verifications necessary. Moreover, most of the existing tools are able to annotate the transcriptome but lack the curation functionalities that are as important as the primary homology-based identification of candidate genes.

As mentioned previously we developed a pipeline called MassBlast (Veríssimo et al. *submitted*), to improve and accelerate the annotation procedure of RNA-seq data. Besides allowing for operators to query multiple databases for performing homology searches, the tool also provides a set of parameters that can be defined in a user-friendly way. MassBlast seeks the best alignment between the sequences in a dataset consisting of contigs obtained from RNA-seq assemblies and a database of known sequences. In the context of BacHBerry and as a general validation of the method, MassBlast was used for the analysis of the blackberry transcriptome, and preliminary results showed a significant improvement in annotation speed (five minutes versus at least 26 working days, to annotate all known CYP families in blackberry) with very high accuracies. MassBlast is freely available at https://github.com/averissimo/mass-blast/.

Sequence design for heterologous expression

643 Synthetic biology aims at constructing microorganisms capable of producing economically-relevant
644 chemical products in a cost-effective way, which involves optimizing the functioning of metabolic pathways
645 and reactions. These pathways and reactions involve proteins that are frequently heterologous. In order to
646 increase the production of metabolites, it is possible to optimize heterologous protein production in the target
647 host. This may be done by altering the codons in the gene that encodes for that protein. Each codon is a
648 sequence of three nucleotides encoding one amino acid that is part of a protein. Since each amino acid can be
649 encoded by several synonymous codons, a given protein can be expressed using a large number of different
650 codon combinations.

651 To address this problem, we worked on improving the machine learning models capable of predicting protein
652 expression levels based on their codon usage frequency (Fernandes et al. 2016) by using Support Vector
653 Regression (SVR) and Partial Least Squares (PLS). SVR yields predictions that surpass those of PLS. It has
654 been shown that it is possible to improve the predictive ability of the models by using two additional input
655 features, namely codon identification number and codon count, besides the already used codon bias and
656 minimum free energy. In addition, applying ensemble averaging to the SVR or the PLS model improved the
657 results even further. Different ensembles and features can be tested with the aim of further improving the
658 prediction models.

659 *Metabolic network optimization*

660 Another goal of the computational activities of BacHBerry was to explore methods for *in silico* metabolic
661 engineering of phenolic production in *L. lactis* and *C. glutamicum*. For this we selected four model target
662 compounds: fisetin, *trans*-resveratrol, pelargonidin and quercetin. Additionally, optimization of malonyl-
663 CoA production was also considered, since this is known to be a major bottleneck in heterologous flavonoid
664 production. The first step involved performing network optimization for a single population, which included
665 prediction of knock-out strategies and determination of minimal sets of genetic interventions that could
666 guarantee an increased production of the target compound production based on stoichiometric models for the
667 two organisms. These predictions were compared with experimental results and model refinement was

conducted in cases of discrepancies. For the second step, we considered a use of microbial consortia for production purposes. A method was developed that is being used for modeling the production of the compounds of interest for BacHBerry using *L. lactis* and *C. glutamicum*. Other, possibly larger consortia, may be considered in the future.

Single strain-based production

Recently, several computational methods for *in silico* metabolic network optimization have emerged and are currently being applied in metabolic engineering (Vera et al. 2010; Zomorrodi et al. 2012; Machado et al. 2015; Simeonidis and Price 2015; Bergdahl et al. 2015). They are based on different approaches and rationales, thereby leading to distinct solutions. One key issue is the multitude of results, known to be dependent on the specific algorithms and solvers, used. In order to address this problem, a consensus-based approach was developed, following our work on meta-analysis of transcriptomic studies (Caldas et al. 2014). This method is based on running several optimization procedures and analyzing *a posteriori* the solutions, looking for a consensus. The rationale is to have rankings of hypotheses that may provide confidence in particular sets of proposed genetic alterations from various aspects.

In the case of a single population of microbes (*i.e.* a monoculture), the genome-scale models of *L. lactis* and *C. glutamicum* were extended with the biosynthetic pathways of the four above-mentioned target compounds (**Figure 9**). Subsequently, five different optimization methods were applied to the genome-scale models (extended with the heterologous pathways): OptKnock (Burgard et al. 2003), MOMA (Segrè et al. 2002), OptGene (Patil et al. 2005), RoboKod (Stanford et al. 2015), and RobustKnock (Tepper and Shlomi 2010), and the consensus ranking was obtained for each strain and compound. The hypotheses were generated with the rank product test and the outputs were lists of gene deletions that would achieve the best ranks using defined criteria, namely maximum predicted target compound production, maximizing the minimum predicted target compound production and distance from the wild-type flux distribution. These results were then further filtered and experimentally validated, resulting in several promising mutant strains where

692 production was increased by up to three-fold. Implementation of this pipeline is possible via a software
693 package called OptPipe, available at https://github.com/AndrasHartmann/OptPipe.

694 We then explored the concept of multi-objective optimization in the field of metabolic engineering when
695 both continuous and integer decision variables were involved in the model. In particular, we proposed multi-
696 objective models to suggest reaction deletion strategies and to deal with problems where several functions
697 must be optimized simultaneously, such as maximization of bioproducts while minimizing toxicity. We thus
698 introduced Multi-Objective Mixed integer Optimization for metabolic engineering, a computational
699 framework that aims to model and solve optimization problems, generated for predicting reaction knockout
700 strategies by means of multi-objective programming. We compared the results from our method with those
701 obtained by using the well-known bi-level optimization model (OptKnock). Furthermore, we studied two
702 multi-objective optimization problems arising from the metabolic engineering of microorganisms, showing
703 that indeed a multi-objective setting is promising, as it expands the set of generated hypotheses and allows
704 inclusion of multiple goals, simultaneously.

705 For a community of microbes

706 Pure microbial cultures have long been used for production of high-value compounds, as illustrated by the
707 example of *S. cerevisiae* producing artemisinic acid, an important precursor for the anti-malaria drug
708 artemisinin, on a large scale (Ro et al. 2006; Lenihan et al. 2008). However, there is increasing evidence that
709 such processes could run more efficiently if a community of microbial species is used (Logan and Rabaey
710 2012; Jones et al. 2016; Wen et al. 2016). There are multiple reasons including an increased production
711 efficiency of the system due to lessening of metabolic burden for individual cells. Another important
712 consideration is that such community systems might have higher chances of avoiding toxic effects specific
713 for individual microorganisms. Microbial consortia are believed to be able to sustain more complex
714 production pathways, as well as being more robust as a group. The challenge, however, remains in
715 establishing precisely which set of strains or species is best for the production of any given compound.

As part of BacHBerry, we introduced an initial topological model and a combinatorial algorithm that enabled us to propose optimal consortia to produce compounds that were either exogenous to the consortium, or were endogenous but where interaction between the species in the consortium could improve the production line (Julien-Laferrière et al. 2016). For initial validations of the model and the method, we applied it to two case-studies taken from the literature. The first case involved production of two bioactive compounds, penicillin and cephalosporin C, both of which are antibiotics used in the pharma industry. A synthetic consortium composed of four different species, three Actinobacteria (*Streptomyces cattleya*, *Rhodococcus jostii* RAH_1 and *Rc. erythropolis* BG43) and one methanogenic Archaea (*Methanosarcina barkeri*), was then run through the algorithm, allowing introduction of genetic manipulations and regulatory processes. We showed that the best solution involved only two of the four species, namely *St. cattleya* and *M. barkeri* (Julien-Laferrière et al. 2016).Another considered case was a consortium that may be seen as artificial in the sense that it associates two wild-type populations of microbes, pathogenic γ-proteobacteria *Klebsiella pneumoniae* and methanogenic Archaea *M. mazei*, which normally do not interact in nature. *K. pneumoniae* is a natural producer of 1,3-propanediol, which is an important building block for polymer production. Our results demonstrated that co-cultivation of *K. pneumoniae* with *M. mazei* may enable obtaining higher yields of this compound in *Klebsiella* (Julien-Laferrière et al. 2016). This supported a previous study involving co-cultures of *Clostridium sp.* with methanogenes such as *Methanosarcina sp.* CHTI55 (Koesnandar et al. 1990). The developed algorithms are currently being used for developing consortia of polyphenol-producing Gram-positive bacteria. This task was inspired by the work of Jones and colleagues, who were able to achieve an overall 970-fold improvement in titers for flavan-3-ols production from phenylpropanoic acids by using a co-culture of two *E. coli* strains with further optimization of cultivation conditions, as compared to previous studies that used monocoltures for this purpose (Jones et al. 2016).

Recovery and purification of polyphenols from fermentation broth

Downstream processing is an important part of any production process and consists of steps required for recovery and purification of biosynthetic products from fermentation broth, which may have a significant impact on the final costs of a product (Goldberg 1997). In the design of downstream processing within

742 BacHBerry, *trans*-resveratrol (**Figure 7**) was used as the test product, being one of the model phenolic
743 compounds that was successfully produced in both *C. glutamicum* and *L. lactis*.

744 In a typical fermentation broth, most of the components present are either hydrophilic (*e.g.*, salts, organic
745 acids, non-consumed carbon sources (usually glucose), nitrogen source (ammonium sulfate, for instance)) or
746 are much larger molecules than *trans*-resveratrol (*e.g.*, proteins, nucleic acids, cells in suspension). This
747 suggested a downstream processing design strategy that used either solubility and hydrophobicity as the
748 driving forces, or alternatively – size exclusion. Since, at low concentrations (around 100 mg l^{-1}),
749 crystallization would involve high energy costs per kg of product recovered, this option was discarded. Two
750 possible extraction techniques, namely *liquid-liquid extraction* and *solid-liquid adsorption*, were investigated
751 as means of recovering and purifying extracellularly produced *trans*-resveratrol from a *C. glutamicum*
752 fermentation broth containing spent CGXII medium and glucose, due to the higher production efficiency
753 found in this bacterium. The downstream process design aimed at a recovery of at least 80% and a purity of
754 95% *trans*-resveratrol.

755 *Liquid-liquid extraction* is a technique to separate a given compound from a feed solution using an
756 immiscible auxiliary phase that should be selective for that compound (Cox and Rydberg 2004). The
757 logarithm (base 10) of the partition coefficient of *trans*-resveratrol between the aqueous and different
758 potential organic phases was determined experimentally for several different solvents (**Figure 10**). The spent
759 fermentation broth from *C. glutamicum* was used as the aqueous phase in these experiments.

760 All tested organic solvents performed very well in extracting *trans*-resveratrol (minimum log P value is ~0.5,
761 corresponding to a partition coefficient of 3.2), identifying them as suitable candidates for the recovery of
762 this compound (when having different impurities present) (**Figure 10**).

763 One of the drawbacks of using liquid-liquid extraction, however, is that it may interfere with protein folding
764 and stability (Prince and Smith 2004). Besides measuring the partition of the polyphenols of interest in the
765 aqueous and organic phases, the same analysis was performed for the total protein content. Although a
766 considerable part of the total protein remained in the aqueous phase, some of the protein seemed to be

present in between the two liquid phases (probably as a result of denaturation and foaming) (**Figure 11**). This phenomenon might reduce the recovery of the desired phenolics, as it was also observed experimentally (data not shown).

Solid-liquid adsorption using hydrophobic resins is another way of purifying *trans*-resveratrol. As in liquid-liquid extraction, the high hydrophobicity of *trans*-resveratrol can be used to recover it selectively from the fermentation broth, leaving the more hydrophilic impurities behind. After an initial resin screen, one of the resins selected was the polystyrene-divinylbenzene based Amberlite XAD-16 from Sigma-Aldrich (particle diameter: 250-841 µm; dipole moment: 0.3; surface area: 900 m^2 g^{-1}; pore diameter: 100 Å).

To test the ability of this resin to recover *trans*-resveratrol from *C. glutamicum* fermentation broth (containing CGXII spent medium and glucose), a lab-scale experiment was performed with an AKTA Explorer chromatographic workstation (GE Healthcare, Sweden) (see **Figure 12**). A 30 ml column was packed with resin and pre-equilibrated with MilliQ water. Afterwards, 10 ml of sample was injected and a washing step with pure water was performed for 10 column volumes (CV). During elution, a gradient of water/ethanol was run for about 12 CV and the peak of *trans*-resveratrol showed up at around 18 CV, at an ethanol percentage of 50% (v/v). Regeneration was performed with 100% ethanol.

More hydrophilic molecules such as organic acids, sugars and salts appeared in the flow-through (the first 10 CV). During the water/ethanol gradient, molecules desorbed as a function of their hydrophobicity. More hydrophobic molecules, such as *trans*-resveratrol, are supposed to elute at higher ethanol content in the mobile phase. In this case, *trans*-resveratrol appeared to be the compound eluting between 16 and 20 CV, since it also showed absorption at 304 nm (Camont et al. 2009).

Although both liquid-liquid extraction and solid-liquid adsorption present their own advantages and disadvantages, a strong point for using adsorption is the use of ethanol instead of any other organic solvent that might not be regarded as GRAS. Moreover, not using liquid-liquid extraction avoids any possible problems related with protein precipitation and emulsification. The ethanol used during elution in adsorption

needs to be recovered from water by distillation (the azeotrope poses no problem as 80% (v/v) ethanol can be used).

The proposed downstream process started with a solid-liquid separation step by centrifugation to separate biomass from the liquid broth. The clarified liquid phase was then sent continuously to one of two adsorption columns, operating staggered in time. The fraction rich in *trans*-resveratrol was subsequently evaporated, resulting in a wet slurry containing the polyphenol in the solid phase. This slurry was filtered in a rotary vacuum filter and washed with water to remove remaining impurities adhered to the surface of the crystals. The final step was a drying step to obtain the product in its final solid form. All the outlet streams containing ethanol were directed to a distillation column in order to recover the solvent. A make-up stream ethanol was added to compensate for losses.

Optimization of phenolic production by fermentation and scale-up of bioprocesses

There are multiple differences between microbial cultivation in shake flasks versus in bioreactors. The former lacks monitoring and control of various parameters, such as dissolved O_2 concentration and pH (Zelle et al. 2010). Furthermore, the fed-batch fermentation mode, where one or more nutrient is being supplied throughout cultivation (Moulton 2014), can be done in a controllable and reproducible fashion only in bioreactors. These properties make bioreactors a suitable stage for optimization of cultivation conditions, often leading to significant improvements in production yields (Hujanen et al. 2001; Ratnam et al. 2003; Li et al. 2010; Rani and Appaiah 2011).

From the beginning of the project it was clear that optimization of fermenter operation would need to be carried out using a model compound, because any newly-identified compounds would only be available at the end of the project. Hence this section of the project was focused on optimization of production of the model compound *trans*-resveratrol. However, it was expected that the strategies defined and the conclusions extracted from these results on *trans*-resveratrol will be valid for the optimization of the fermentation processes for the production of other target compound. Several *L. lactis* and *C. glutamicum* strains designed within the project were evaluated and used for fermenter process optimization. The experiments were carried

816 out in agitated 2 to 5 litre fermenters with automatically controlled pH, dissolved oxygen and temperature.
817 Whenever required, oxygen was transferred to the medium by air injection at the bottom of the vessel.

818 A major goal of the project was the development of an economically feasible and sustainable industrial
819 process, therefore the following key optimization criteria were defined: (a) minimisation of the operation and
820 investment cost and (b) maximisation of the production rate and operation yield. To achieve these
821 optimization goals, the following variables have been investigated: (i) fermentation medium composition, (ii)
822 dissolved oxygen concentration, (iii) operation strategy (batch vs. fed-batch and integrated vs. separate
823 downstream operation). Our experiments suggested that there were several constraints limiting the operation
824 strategy to be implemented, namely: cell metabolism, the stability of *trans*-resveratrol inside the fermenter,
825 and the affinity of *trans*-resveratrol for the adsorption medium and solvents compared to other compounds
826 present in the fermentation broth.

827 Medium composition is an important parameter that often has a significant contribution towards the final
828 production costs. According to our initial experiments, supplementation of *C. glutamicum* minimal medium
829 with Brain-Heart Infusion (BHI) and a fatty-acid biosynthesis inhibitor, cerulenin, had a strong positive
830 effect on the final titers of *trans*-resveratrol. However, removing these two components from the medium
831 reduced medium costs by approximately 300-fold, resulting in a more than 50-fold drop in production costs
832 (267 €/g compared to 5 €/g of *trans*-resveratrol without supplementation of BHI and cerulenin).

833 We also evaluated the feasibility of producing phenolic compounds from waste materials, namely cheese
834 whey using *L. lactis* as a host. Whey is a lactose-rich dairy industry by-product with a high environmental
835 impact, if dumped untreated (Palmieri et al. 2017). To achieve this task, our production strain was modified
836 to allow it to grow and produce *trans*-resveratrol from lactose by introducing a plasmid carrying the *lac*
837 operon (Maeda and Gasson 1986). Production at 2 litre-scale was tested using lactose as carbon source and
838 comparing the productivity between the cultures with glucose or lactose. These experiments demonstrated
839 that the production of *trans*-resveratrol from lactose, and therefore from whey, is possible with slightly
840 inferior productivity compared to glucose. However, if whey is chosen as the fermentation medium, other,

841 more important, issues arise. The cost of whey is comparable to the cost of glucose but the cost of the
842 fermentation medium is only a small part of the total production costs, so variables such as whey availability
843 and quality, as well as the stability of supply and collecting logistics will determine the feasibility of the
844 operation more than the small losses in productivity. Clearly, if a phenolic production industrial unit were to
845 be implemented inside a dairy company with excess whey, the whey might then be the best raw material.

846 Another parameter investigated was the influence of dissolved oxygen concentration on the production
847 efficiency of *L. lactis*. This bacterium can activate its respiratory chain in the presence of hemin in the
848 culture medium, and this has been demonstrated to enhance its survival allowing it to grow to higher cell
849 densities (Duwat et al. 2001). Therefore the production strain efficiency was tested under the following
850 conditions: aerobic with hemin added to the fermentation broth, aerobic without hemin, semi-aerobic, and
851 fully anaerobic. In line with the available data, there was a significant increase in the final biomass when the
852 strain was grown under aerobic conditions with hemin. However, the opposite was observed for the
853 production of *trans*-resveratrol where the highest amounts of the compound were detected under anaerobic
854 conditions.

855 Lastly, we have also worked on establishing a system for continuous product removal from the fermentation
856 broth in order to simplify downstream processing and reduce *trans*-resveratrol oxidation due to prolonged
857 incubation. Two strategies for continuous adsorption and storage of secreted *trans*-resveratrol in a resin have
858 been devised and tested: (i) "*in situ*" product removal that consisted of adding a resin directly to the
859 fermentation broth and (ii) "continuous product removal" (ConPR) that involved constant pumping of the
860 fermentation broth through a column filled with the adsorption resin. The supernatant was separated from
861 cells via a tangential flow micro-filtration device, sent though the column, and then returned back into the
862 fermenter (**Figure 13**).

863 The obtained results demonstrated that both the "*in situ*" and ConPR strategies lowered the *trans*-resveratrol
864 productivity, probably due to co-adsorption of *p*-coumaric acid, the precursor for *trans*-resveratrol. This
865 effect was most pronounced in the "*in situ*" operation mode due to continuous contact of the resin with the

supernatant and cells. In the ConPR operation mode the effect was reduced due to low supernatant flow rate through the resin, however adsorption of *trans*-resveratrol was also reduced. In contrast, in the "*in situ*" mode the amount of adsorbed end-product was nearly identical to the amount of extracellular and intracellular *trans*-resveratrol produced in control experiments without addition of resin. Although the two techniques did not increase the overall productivity, they still simplified the downstream processing and may lead to a production cost reduction by eliminating the need to break down the cells. However, future research may provide a solution to avoid precursor co-adsorption resulting in increased polyphenol productivity.

Dissemination, training, societal and ethical issues

In addition to applying the latest technological advances from life sciences for establishing a pipeline for microbial-based polyphenol production, BacHBerry analyzed the economic potential of the possible products developed from this research. The assessment of economic issues of the project has been focused on the market potential of the natural colorants identified during the screening of the germplasm collection. This was done along with obtaining an overview of the relevant regulations and procedures for approval of novel food additives, including colorants, in the EU, the United States (US), and China. Regulations within both the EU and China confer a strict set of rules for bringing a novel food colorant, even a natural one, to the market. These rules consider not only the composition and purity of the substance, but also the process through which the compounds have been produced. In contrast, in the US, a new food additive can gain access to the market by simply passing the GRAS assessment. Hence, if a novel polyphenolic colorant could be produced using engineered microbial cell factories, the US market should probably be the first market to be targeted, owing to less strict regulatory requirements for the approval.

Another topic addressed in BacHBerry was access and benefit sharing of genetic resources. As the project was built upon screening of berry species from multiple countries, including China, Russia, Chile, Portugal, and the UK, it is important to harness these resources in a responsible manner. *The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization* (NP-ABS) is an agreement consisting of a set of rules for fair sharing of monetary and non-monetary benefits

arising from the usage and commercialization of genetic resources and traditional knowledge associated with those. It aims at achieving fairness and equity of gain distribution, especially towards indigenous and local communities (SCBD 2011; Morgera et al. 2014). The Protocol came into force in the EU as of November 20th 2016. As most of the BacHBerry project partners were from EU Member States, we began collecting information about the best practices suggested from both the Convention on Biological Diversity (CBD) and the EU, and kept monitoring the relevant guidelines and updates from the EU on implementing ABS. Among the home countries of the project partners China, Denmark, France, Germany, Switzerland, and UK ratified or accepted the NP-ABS (as of November 2016) and began implementing the aforementioned regulations (European Parlament 2014). The UK also made its own regulation on implementing the Protocol (European Parlament 2014; NMRO (UK) 2015; Secretary of State (UK) 2015).

Funded under the theme of "Knowledge-based Bio-Economy", a final aim of the project was to provide scientific and technical solutions to harness berry resources, as well as to contribute to sustainable development of the economy. We consequently investigated the possible contributions of our research to build a more sustainable society based on environmental, economical, and social impacts (Pei and Schmidt 2016). Furthermore, we conducted several open dialog events, namely workshops and interviews with potential stakeholders from Austria, Denmark, and China. One of the surprising outcomes was that the general public raised concerns over the use of genetically-modified organisms (GMOs) in the pipeline a lot less frequently than originally expected. A free, science-based game for mobile devices called "Berry Maker" was produced (http://www.berrymaker.com). The game allows the user to follow the pipeline designed within our project, from acquisition of diverse berries, to extraction and analysis of biosynthetic genes, to microbial fermentation-based production of specific polyphenols and running a biotech factory that is exposed to real world situations, such as price fluctuations. The game is also available on iTunes[1] and Google Play[2] store. Lastly, a 22 minute documentary film was produced, showing sampling of berries,

[1] See: https://itunes.apple.com/us/app/berrymaker-dna-soda-factory/id1163115116?ls=1&mt=8

[2] See: https://play.google.com/store/apps/details?id=com.biofaction.berrymaker

analysis of interesting compounds, and experiments with natural color pigments for food products from across Europe (see: https://vimeo.com/193467652).

Conclusions

During three years of the project, the BacHBerry partners successfully established a complete pipeline for screening, bioprospecting, identification, and microbial production of plant phenolic compounds in Gram-positive bacteria. A summary of the most important results is presented in **Table 5**. The major highlights are construction of the extensive germplasm collection database, establishment of a method for high-throughput screening of bioactive extracts using the yeast-based human disease models, generation of a vast berry transcriptome database, construction of microbial cell factories in *L. lactis* and *C. glutamicum* for the production of polyphenols with yields comparable to those currently available from the literature, release of several software packages for transcriptome analysis and *in silico*-based metabolic engineering, as well as optimization of fermentation conditions and downstream processing for the selected organisms aimed at increasing the yield and the purify of the final products. The pipeline has been completely validated and is available to be picked up by the biotech industry for further improvement and commercialization. The developed methods and protocols should be suitable for discovery, as well as production of most groups of polyphenols in the above-mentioned organisms, without a need for significant alterations and adaptations. Overall, this project provided the scientific community with enabling tools and resources to further advancing research on berries, phenolic compounds, and metabolic engineering of microorganisms. Additional efforts were also focused on communication with stakeholders, public outreach, and popularization of science among the younger generation. The majority of the project outcomes is already available to the scientific community and the industry in form of peer-reviewed articles, or will be published within the coming year.

Acknowledgments

938 The authors would like to thank the European Union's Seventh Framework Programme (BacHBerry, Project
939 No. FP7-613793, and FP7-PEOPLE-2013-COFUND, Project No. FP7-609405) for the financial support.
940 AD, RF, MHHN, PG, SGS, and JF would also like to acknowledge the Novo Nordisk Foundation. We
941 express our gratitude to Dr. Martha Cyert (Stanford School of Medicine, EUA), Dr. Hitoshi Shimoi (National
942 Research Institute of Brewing, Japan) and Dr. Yoshio Araki (Graduate School of Biosphere Science,
943 Hiroshima University, Japan) for providing the yeast strain YAA5. We also thank Dr. Ian Macraedie, RMIT
944 University, Australia) for providing the plasmid p416_GPDpr-GFP-A42.

945

946 **Abbreviations**

947 4CL, 4-coumaryl-CoA ligase; AD, Alzheimer's Disease; ANS, anthocyanidin synthase; ALS, amyotrophic
948 lateral sclerosis; anthocyanidin synthase; ANR, anthocyanidin reductase; C3'H, *p*-coumaroyl
949 shikimate/quinate 3'-hydroxylase; C4H, cinnamate 4-hydroxylase; CBD, Convention on Biological
950 Diversity; CaN, calcineurin; CDRE, Calcineurin-Dependent Responsive Element; CHI, chalcone isomerase;
951 CHR, chalcone reductase; CHS, chalcone synthase; CPR, cytochrome-P450 reductase; CVs, column
952 volumes; CYP, cytochromes P450; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3'H,
953 flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3'-5'-hydroxylase; FLS, flavonol synthase; FNS, flavone
954 synthase; FP7, European Commission's the Seventh Framework programme; GFP, Green Fluorescent
955 Protein; GRAS, generally regarded as safe; HCT, hydroxycinnamoyl-CoA shikimate/quinate
956 hydroxycinnamoyltransferase; HD, Huntington's Disease; HPLC, high-performance liquid chromatography;
957 LAR, leucoanthocyanidin reductase; LC-MS, liquid-chromatography mass-spectrometry; NDs,
958 Neurodegenerative diseases; NFAT, Nuclear Factor of Activated T-cells; NFκB, Nuclear Factor κB; NP-
959 ABS, The Nagoya protocol on access to genetic resources and the fair and equitable sharing of benefits
960 arising from their utilization; OMT, *O*-methyltransferase; PAL, phenylalanine ammonia-lyase; PCA,
961 principal component analysis; PD, Parkinson's Disease; PLS, partial least squares; RNA-seq, RNA

962 sequencing technology; STS, stilbene synthase; SVR, support vector regression; TAL, tyrosine ammonia-
963 lyase; UFGT, flavonoid 3-*O*-glucosyltransferase.

964

965 **Appendices**

966 Supplementary_Materials.docx

967 Supplementary_Tables.docx

968

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Figure legends

Figure 1: **a)** The molecular structure of the stilbene *trans*-resveratrol. **b)** Basic flavonoid structure. R_x are positions where decorating groups (*e.g.* hydroxyl, acyl, glycosyl, etc.), which differentiate the compounds and alter their chemical properties, could be attached.

Figure 2: Principal component analysis (PCA) plots of the full dataset without reference material and blanks. Each dot represents the profile of one sample aliquot whereas lines delimit groups of samples that cluster together. **a)** PCA plots of the full dataset without reference material and blanks in positive mode. Components (Scr) 1 and 2 explain 9.79% and 7.59% of the variation respectively. **b)** PCA plots of the full dataset without reference material and blanks in negative mode. Components (Scr) 1 and 2 explain 13.20% and 10.30% of the variation respectively.

Figure 3: Schematic representation of yeast models. a. Yeast models of neurodegenerative diseases. Humanized yeast strains encoding chimeric fusions of disease genes with GFP (green fluorescent protein) under the control of a galactose-inducible (GAL1) promoter. Expression of Aβ42, αSyn (SNCA gene), FUS and HTTpQ103 leads to the formation of toxic aggregates, recapitulating the pathological processes of Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), respectively. b. Yeast model of Crz1 (NFAT orthologue) activation. Crz1 is activated by the serine/threonine protein phosphatase calcineurin (composed by CnA and CnB subunits). The reporter strain encodes the *lacZ* gene under the control of a promoter containing Crz1-binding sites, allowing the assessment of Crz1 activation through the measurement of β-galactosidase activity.

Figure 4: Identification of bioactivities in the berry germplasm. a) Strategy used for the discovery of bioactive compounds for Alzheimer's disease. BY4741_*erg6* recombinant yeast cells expressing GFP-Aβ42 were grown in SD galactose medium and cell viability was assessed by growth curves for 24 h in cells challenged or unchallenged with *Lycium chinense* polyphenol extracts. Cells containing the empty vector were used as controls. The areas under the curve (AUC) were integrated using the Origin software

(OriginLab, Northampton, MA). Values represent the mean ± standard error of mean (SEM) of at least three biological replicates.

b) Strategy used for the discovery of bioactive compounds with anti-inflammatory potential. BY4741 yeast cells encoding $CDRE_{promoter}$-*lacZ* were treated or not with *Lycium chinense* polyphenol extracts and induced with 1.8 mM $MnCl_2$. Crz1 activation was assessed by monitoring β-galactosidase activity using ortho-nitrophenyl-β-galactoside (ONPG). The immunosuppressant FK506, a well-known inhibitor of calcineurin (Dumont 2000) was used as a positive control. The values represent the mean ± SEM of at least three biological replicates, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 5. Yeast as a discovery platform of therapeutic compounds. Iterative bio-guided fractionation of complex mixtures allows the discovery of small molecules improving cell growth, by rescue of protein aggregates toxicity, or inhibiting inflammatory processes. High-throughput formats aligned with chemical identification by LC-MS approaches accelerate the identification of candidate compounds.

Figure 6: Polyphenol biosynthetic pathway in plants (modified from Falcone Ferreyra et al. 2012). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3’5’H, flavonoid 3’-5’-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT, flavonoid 3-*O*-glucosyltransferase. LAR, leucoanthocyanidin reductase; FNS, flavone synthase; FLS, flavonol synthase; STS, stilbene synthase. P450 enzymes are highlighted in red.

Figure 7: Overview of the endogenous phenylpropanoid degradation and the engineered pathway for polyphenol synthesis in *C. glutamicum*. One of the precursors for the production of the stilbene *trans*-resveratrol (catalyzed by stilbene synthase, STS) or for naringenin chalcone (catalyzed by chalcone synthases, CHS) is *p*-coumaroyl-CoA. Naringenin chalcone isomerizes to the (2*S*)-flavanone naringenin either spontaneously or catalyzed by the activity of chalcone isomerase (CHI). In *C. glutamicum*, *p*-coumaric acid can be degraded to 4-hydroxybenzoate by a CoA-dependent, β-oxidative deacetylation pathway. 4-Hydroxybenzoate is subsequently hydroxylated to protocatechuate, which is catabolized to succinyl-CoA

and acetyl-CoA by the β-ketoadipate pathway. Genes coding for the underlined enzymes were deleted in the course of the construction of the platform strain *C. glutamicum* DelAro[4].

Figure 8: Heterologous biosynthetic pathway for fisetin assembled in *E. coli*. TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl-CoA ligase; CHS::CHR, chalcone synthase::chalcone reductase fusion; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3'H::CPR, flavonoid 3'-hydroxylase::cytochrome P450 reductase fusion.

Figure 9: Heterologous biosynthetic pathways introduced into the genome-scale models of *C. glutamicum* and *L. lactis* leading to the four target compounds: i) *trans*-resveratrol ii) pelargonidin iii) quercetin and iv) fisetin. Two heterologous enzymes (TAL and 4CL) are common for the biosynthesis of the phenolic molecules. Enzyme abbreviations: TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl-CoA ligase; STS, stilbene synthase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; F3H, flavanone 3–hydroxylase; FLS, flavonol synthase; F3'H, flavonoid 3' hydroxylase; CPR, cytochrome P450 reductase.

Figure 10: Partition coefficients in the form of log P values of *trans*-resveratrol using different organic solvents using *C. glutamicum* fermentation broth as the aqueous phase.

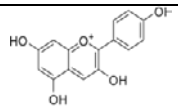
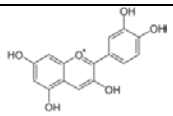
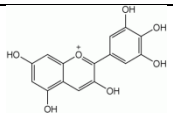
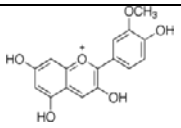
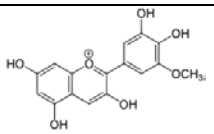
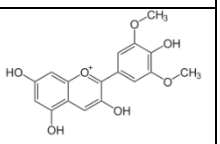
Figure 11: On the left, protein concentration (expressed as percentage of the initial broth protein concentration) in the organic and aqueous phases, after performing liquid extraction. The concentration in the organic phase is almost zero (considering the error bars), indicating that some protein stayed in the interphase. On the right, the formation of an interphase after the broth had been extracted with organic solvent (a mixture of heptanoic acid and hexyl acetate in this case) was clear.

Figure 12: Chromatogram following a pulse injection of 10 ml of *C. glutamicum* spent fermentation broth in a 30 ml column packed with Amberlite XAD-16 resin. A washing step was performed with 10 column volumes (CVs) of MilliQ water. Afterwards, elution was performed with a water/ethanol gradient for 12 CV, followed by a final regeneration step with 100% ethanol.

Figure 13: Experimental set-up for the “Continuous Product Removal” mode.

Tables

Table 1: Anthocyanidin structures (aglycone form) found in different berry species. The presence of a compound is noted with + whereas the absence is noted with -.

Type	Pelargonidin	Cyanidin	Delphinidin	Peonidin	Petunidin	Malvinidin
Structure						
Molecular formula	$C_{15}H_{11}O_5^+$	$C_{15}H_{11}O_6^+$	$C_{15}H_{11}O_7^+$	$C_{16}H_{13}O_6^+$	$C_{16}H_{13}O_7^+$	$C_{17}H_{15}O_7^+$
Fragaria[1]	+	+	-	-	-	-
Ribes[1]	+	+	+	-	+	-
Rubus[1]	+	+	-	-	-	-
Vaccinium[1]	-	+	+	+	+	+
Vitis[1]	-	+	+	+	+	+
Lonicera[2,5]	+	+	+	+	-	+
Lycium[3]	-	-	+	-	+	+
Berberis[4]	-	+	+	+	+	+
Ugni[4]	-	+	-	+	-	-
Aristotelia[6]	-	+	+	-	-	-

[1] Phenol explorer database (http://phenol-explorer.eu); [2] (Chaovanalikit et al. 2004); [3] (Zheng et al. 2011); [4] (Ruiz et al. 2010); [5] (Wang et al. 2016b); [6] (Fredes et al. 2014)

1493 **Table 2:** Selected species for bioactivity testing according to the phytochemical diversity.

Species	**Variety/line**	**Species**	**Variety/line**
Aristotelia chilensis (Molina)Stuntz	-	*Rubus armeniacus* Focke	Himalayan Giant
Berberis buxfolia Lam.	-	*Rubus occidentalis* L.	Huron
Lonicera caerulea L.	S322-23	*Rubus loganobaccus* L.H.Bailey	Sunberry
Lycium chinense Mill.	-	*Rubus loganobaccus* L.H.Bailey	Tayberry
Ribes grossularia L.	9294	*Rubus bartonii* Newton	Ashton Cross
Ribes grossularoides Maxim.	H1-12-1	*Rubus fruticosus* L. *agg.*	Fantasia
Ribes nevadense Kellog	-	*Rubus brigantinus* Samp.	-
Ribes spp.	644217	*Rubus genevierii* Boreau.	-
Ribes spp.	1126	*Rubus henriquesii* Samp.	-
Ribes spp.	2292-1	*Rubus hochstetterorum* Seub.	-
Ribes spp.	Muravushka	*Rubus sampaioanus* Sudre ex Samp.	-
Ribes spp.	-	*Rubus vagabundus* Samp.	-
Ribes petraeum Wulf.	R2-1-2	*Rubus idaeus* L.	Octavia
Ribes x *saundersii* Jancz.	XB4	*Rubus idaeus* L.	Prestige
Ribes bethmontii Jancz.	XW6	*Ugni molinae* Turcz.	Red Pearl
Ribes rubrum L.	S11-3-36	*Vaccinium uliginosum* L.	-
Rubus spp.	B14	*Vaccinium vitis-idaea* L.	-

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Table 3: Transcriptome resources for berries developed on BacHBerry

Species	Plant material	Total number of raw reads
Aristotelia sp.	ripe berries	397,707,372
Berberis sp.	ripe berries	444,362,698
Corema sp.	leaf	353,604,932
Lonicera sp.	ripe berries	397,214,254
*Ribes sp.*1	ripe berries	336,479,242
*Ribes sp.*2	ripe berries	393,665,630
*Rubus sp.*1	berries (3 stages)	1,040,224,680
*Rubus sp.*2	berries (3 stages)	1,064,858,518
*Rubus sp.*3	ripe berries	505,754,030
*Rubus sp.*4	ripe berries	390,608,452
Ugni sp.	ripe berries	405,024,920
*Vaccinium sp.*1	ripe berries	373,159,882
*Vaccinium sp.*2	ripe berries	375,778,718

Table 4: Statistics on five major enzyme families representing conserved biochemical steps of the phenylpropanoid pathway. Candidates displayed more than 70% sequence coverage to reference query sequences. This work led to the creation of a large dataset of candidates used for pathway elucidation and reconstitution in bacterial hosts.

Enzyme families	Candidates per transcriptome (min-max)
Conserved phenylpropanoid pathway enzymes	109-180
CYPs	100-177
UGTs	70-145
2-OGDs	32-88
O-methyltransferases	8-51
Acyltransferases	23-36

1505 **Table 5:** Summary of the outcomes of the project

Specific application area in project	**Bioprospecting for discovery of novel berry phenolics**	**Characterization of phenolics biosynthetic pathways**	**Bacterial cell factories for production of berry phenolics**	**Fermentation and bioprocess engineering**
General aims of BacHBerry	To tap into the biodiversity of berries from around the globe and of their phenolics content	Identification and characterization of berry phenolics biosynthetic pathways	Design and generation of bacterial cell factories for production of high-value berry phenolics	Implementation of production of high-value phenolics in fermenters up to demonstration scale
Achievements of the project	• Standardization of methodologies for harvesting, extract preparation, phenolics content assessment and fractionation • Metabolomics data from berry species originating from UK, mainland Europe, Russia, Chile, and China was obtained and made available in form of an online database • Implementation of robust assays based on the SMART platform for discovery of bioactivity and functionality	• Transcriptomes of 13 germplasms spanning eight genera, seven families and seven orders were generated and analyzed •New algorithms for functional genomics and improved computational methods for pathway identification were established • Over 4000 candidate genes for various biosynthetic steps, transport and regulation of polyphenol production were identified	• *L. lactis* strains were engineered for stilbene (*trans*-resveratrol and pterostilbene) and anthocyanin production • Production of various stilbenes (*trans*-resveratrol, pinosylvin, and piceatannol), methylated stilbenes (pinostilbene and pterostilbene), flavanones (naringenin, pinocembrin, and eryodictiol), and flavonols (kaempferol and quercetin) was achieved in *C. glutamicum* with yields comparable to those obtained in the model organism *E. coli*. Production titers for the produced	• Cultivation conditions for improved productivity were developed for both *L. lactis* and *C. glutamicum* through optimization of fermentation parameters (batch versus fed-batch operation, substrate and dissolved oxygen concentration, etc.) • Whey was evaluated as an alternative carbon source for polyphenol production in *L lactis* • Multiple bioseparation methods for extraction of produced polyphenols from fermentation broth were evaluated

	• Obtained berry extracts were assayed for bioactivity against several human diseases, and additionally evaluated for other functional uses (eq. food additives, antimicrobials). Bioactivities against Alzheimer's, Parkinson's and Huntington's diseases, Amyotrophic Lateral Sclerosis and inflammation were detected. • Several pure bioactive effector compounds were identified and validated	• Biochemical activities of some of the gene candidates were tested, thus validating the predictions	flavonols are the highest titers obtained in engineered microorganism until today • Polyphenol production was further improved by engineering enhanced precursor supply (eq. L-tyrosine, malonyl-CoA) via rational design, modeling-based prediction, and use of biosensors • Alternative biosynthetic routes were explored though engineering of *trans*-resveratrol production from 4-hydroxybenzoic acid (HBA) in *C. glutamicum* • Computational models were developed allowing predicting a minimal set of modifications needed for improved polyphenol production in both a single-strain population and a consortium of two or more species	• Two possible designs for a system for continuous product removal from the fermentation broth were implemented • Resveratrol production in *C. glutamicum* was scaled up from shake flask to 5 l fermenter to demonstration scale (250 l)

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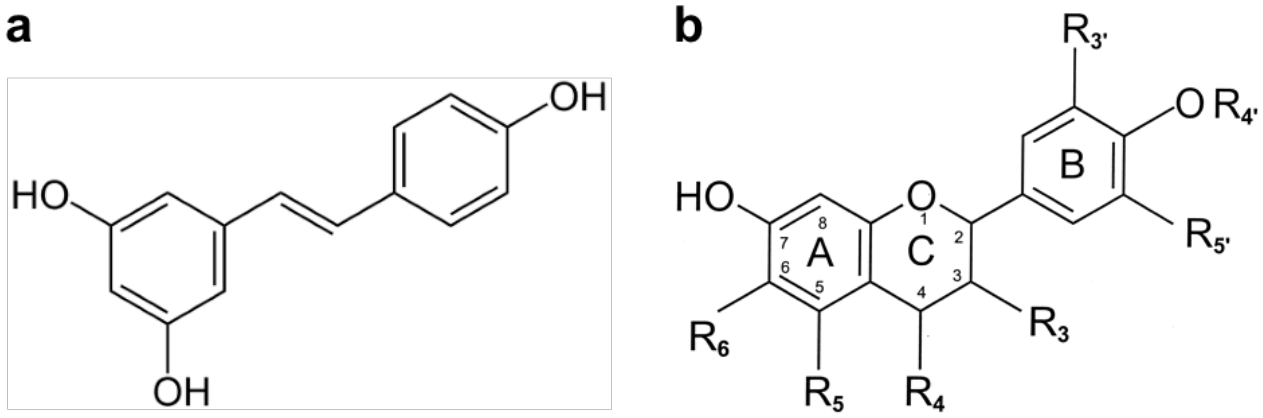

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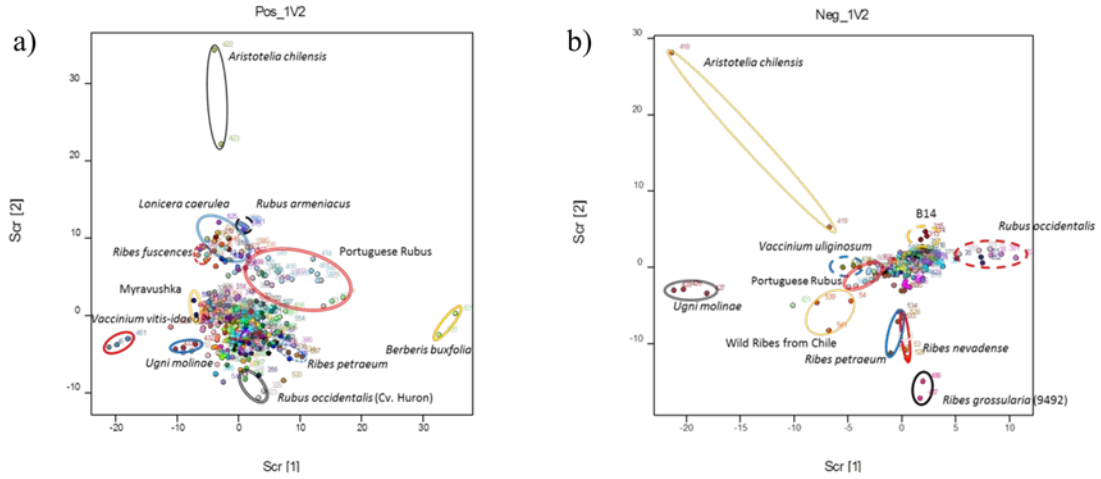

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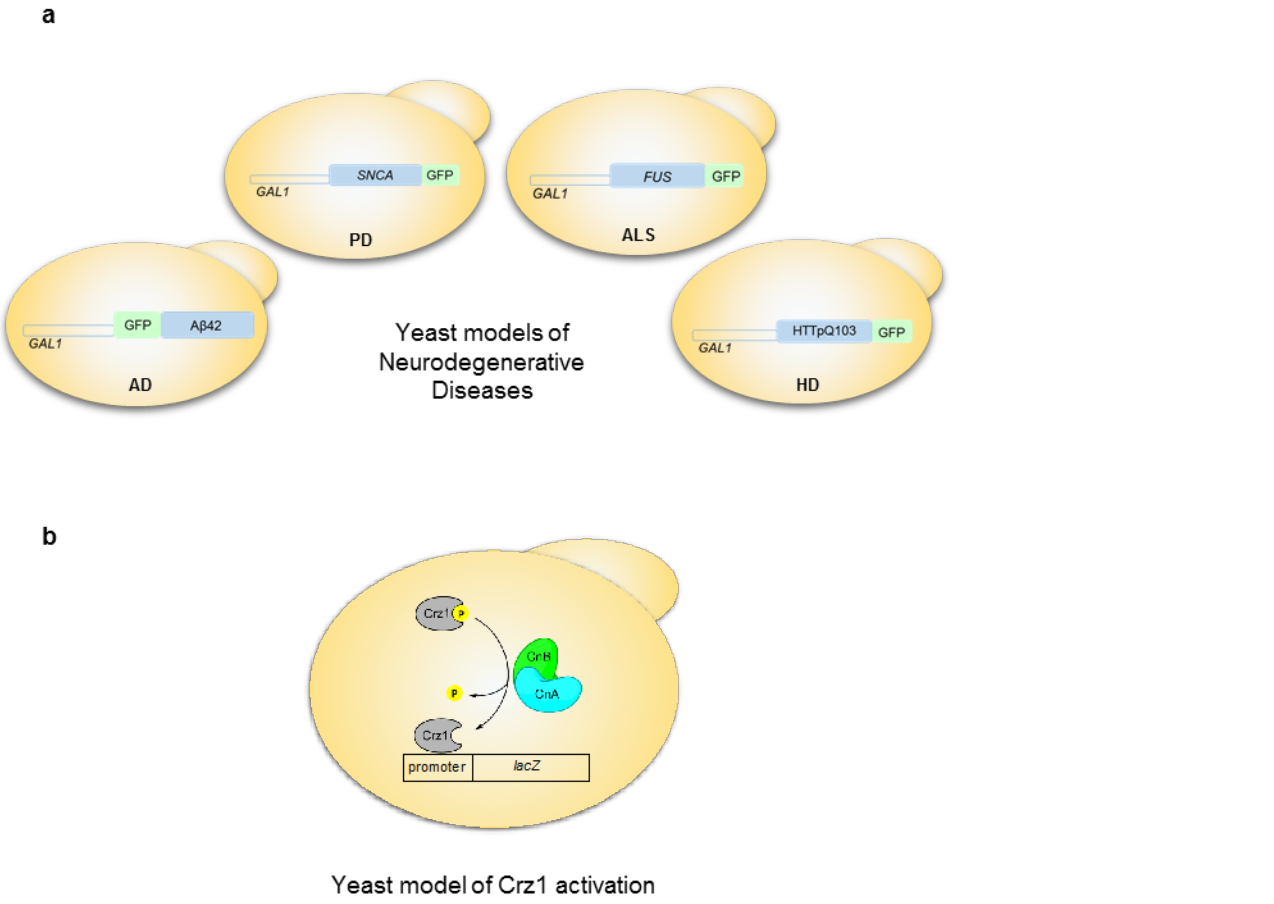

Figure 3: Schematic representation of yeast models. a. Yeast models of neurodegenerative diseases. Humanized yeast strains encoding chimeric fusions of disease genes with GFP (green fluorescent protein) under the control of a galactose-inducible (GAL1) promoter. Expression of Aβ42, αSyn (SNCA gene), FUS and HTTpQ103 leads to the formation of toxic aggregates, recapitulating the pathological processes of Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), respectively. b. Yeast model of Crz1 (NFAT orthologue) activation. Crz1 is activated by the serine/threonine protein phosphatase calcineurin (composed by CnA and CnB subunits). The reporter strain encodes the *lacZ* gene under the control of a promoter containing Crz1-binding sites, allowing the assessment of Crz1 activation through the measurement of β-galactosidase activity.

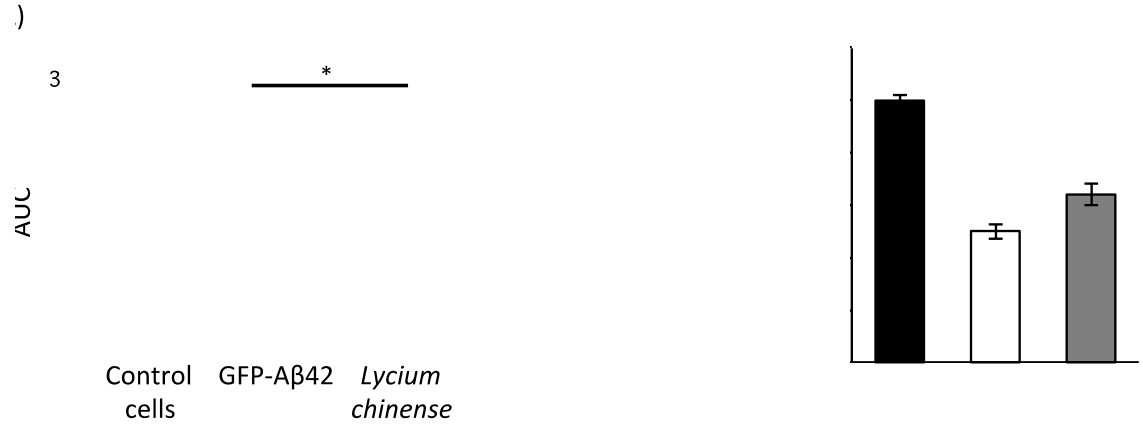

Figure 4: Identification of bioactivities in the berry germplasm. a) Strategy used for the discovery of bioactive compounds for Alzheimer's disease. BY4741_*erg6* recombinant yeast cells expressing GFP-Aβ42 were grown in SD galactose medium and cell viability was assessed by growth curves for 24 h in cells challenged or unchallenged with *Lycium chinense* polyphenol extracts. Cells containing the empty vector were used as controls. The areas under the curve (AUC) were integrated using the Origin software (OriginLab, Northampton, MA). Values represent the mean ± standard error of mean (SEM) of at least three biological replicates.

b) Strategy used for the discovery of bioactive compounds with anti-inflammatory potential. BY4741 yeast cells encoding $CDRE_{promoter}$-*lacZ* were treated or not with *Lycium chinense* polyphenol extracts and induced with 1.8 mM $MnCl_2$. Crz1 activation was assessed by monitoring β-galactosidase activity using ortho-nitrophenyl-β-galactoside (ONPG). The immunosuppressant FK506, a well-known inhibitor of calcineurin (Dumont 2000) was used as a positive control. The values represent the mean ± SEM of at least three biological replicates, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

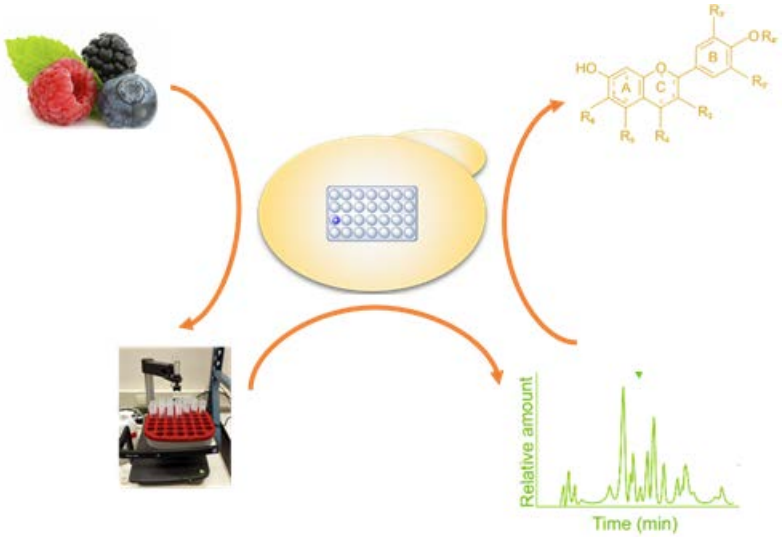

Figure 5. Yeast as a discovery platform of therapeutic compounds. Iterative bio-guided fractionation of complex mixtures allows the discovery of small molecules improving cell growth, by rescue of protein aggregates toxicity, or inhibiting inflammatory processes. High-throughput formats aligned with chemical identification by LC-MS approaches accelerate the identification of candidate compounds.

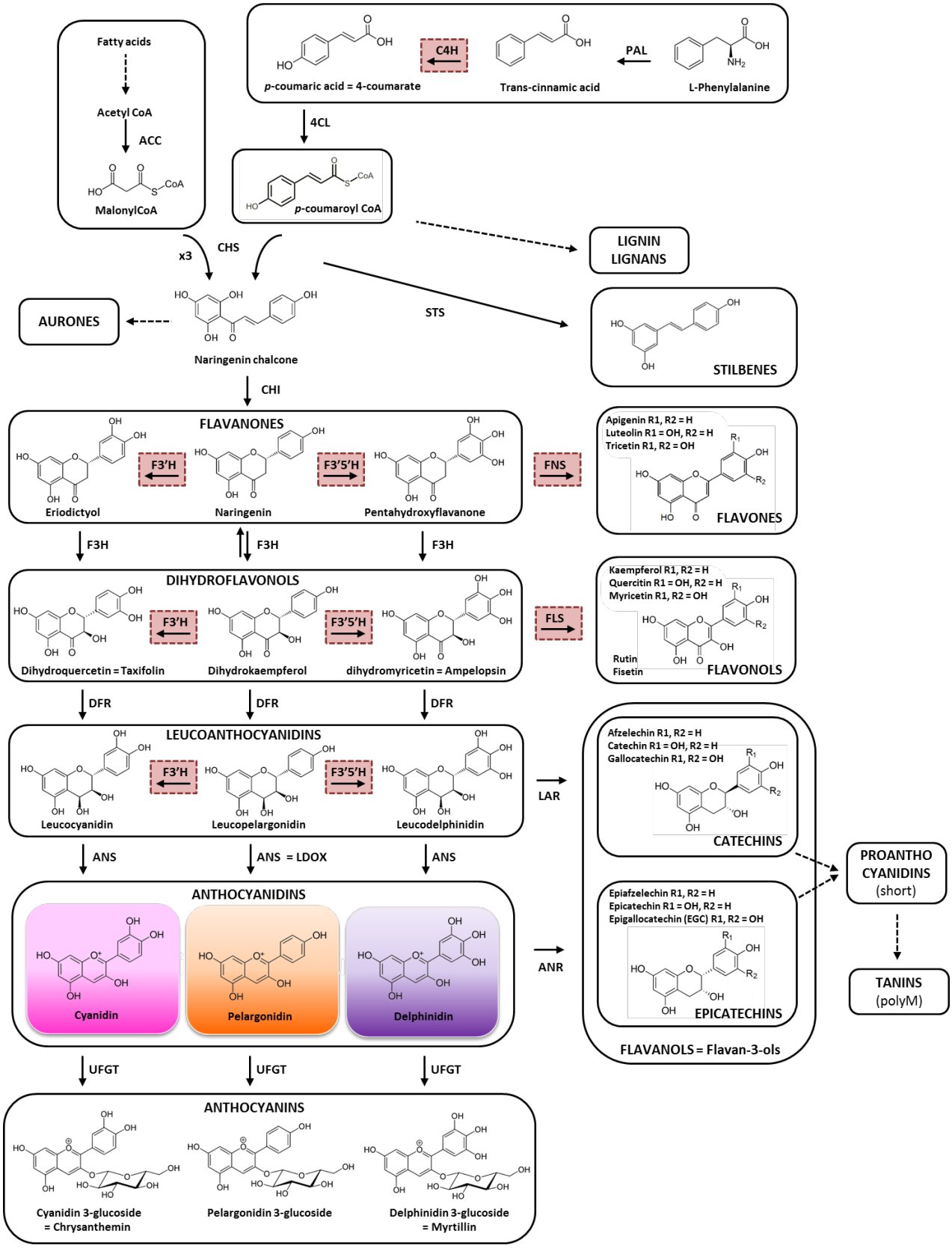

Figure 6: Polyphenol biosynthetic pathway in plants (modified from Falcone Ferreyra et al. 2012). PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaroyl-CoA ligase; CHS,

chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3’5’H, flavonoid 3’-5’-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; UFGT, flavonoid 3-*O*-glucosyltransferase. LAR, leucoanthocyanidin reductase; FNS, flavone synthase; FLS, flavonol synthase; STS, stilbene synthase. P450 enzymes are highlighted.

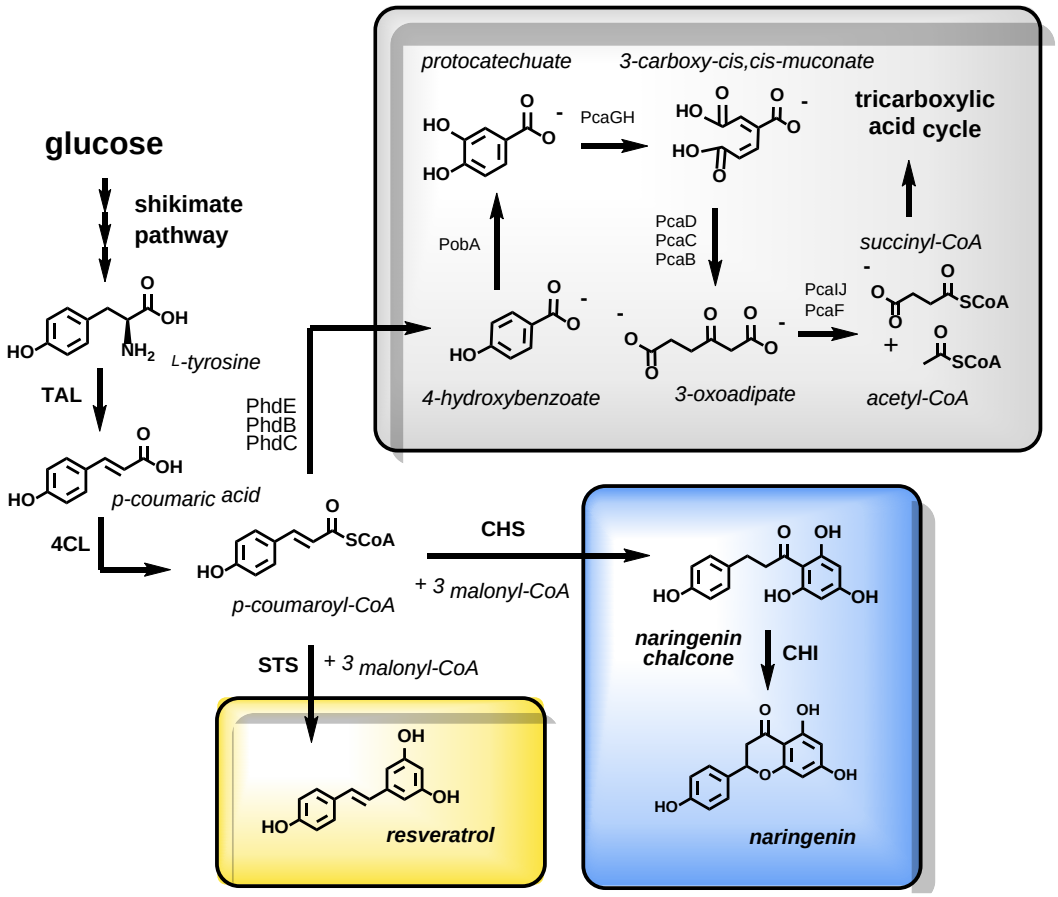

Figure 7: Overview of the endogenous phenylpropanoid degradation and the engineered pathway for polyphenol synthesis in *C. glutamicum*. One of the precursors for the production of the stilbene *trans*-resveratrol (catalyzed by stilbene synthase, STS) or for naringenin chalcone (catalyzed by chalcone synthases, CHS) is *p*-coumaroyl-CoA. Naringenin chalcone isomerizes to the (2*S*)-flavanone naringenin either spontaneously or catalyzed by the activity of chalcone isomerase (CHI). In *C. glutamicum*, *p*-coumaric acid can be degraded to 4-hydroxybenzoate by a CoA-dependent, β-oxidative deacetylation pathway. 4-Hydroxybenzoate is subsequently hydroxylated to protocatechuate, which is catabolized to succinyl-CoA and acetyl-CoA by the β-ketoadipate pathway. Genes coding for the underlined enzymes were deleted in the course of the construction of the platform strain *C. glutamicum* DelAro[4].

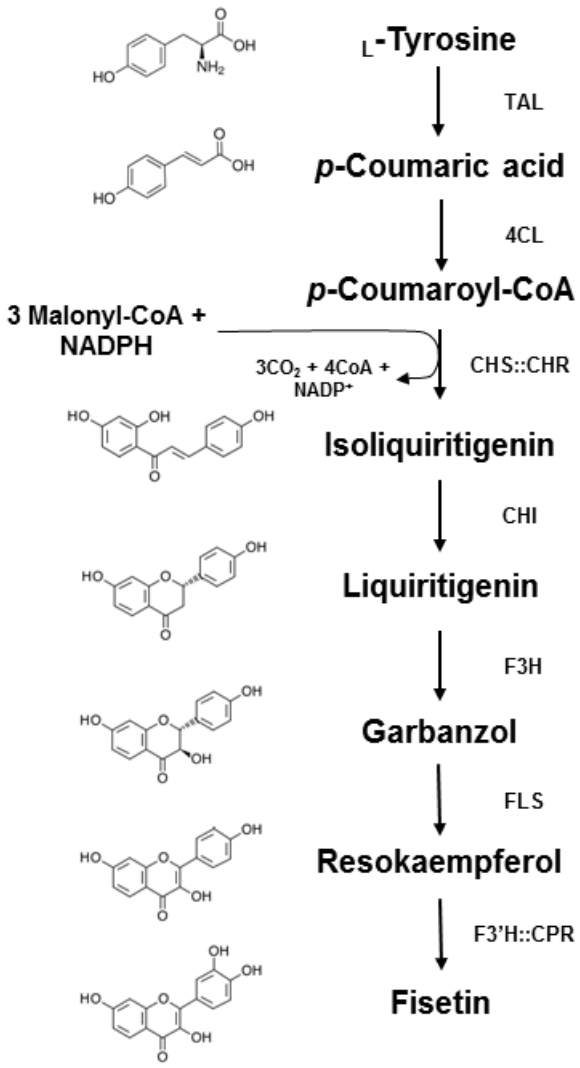

Figure 8: Heterologous biosynthetic pathway for fisetin assembled in *E. coli*. TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl-CoA ligase; CHS::CHR, chalcone synthase::chalcone reductase fusion; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3'H::CPR, flavonoid 3'-hydroxylase::cytochrome P450 reductase fusion.

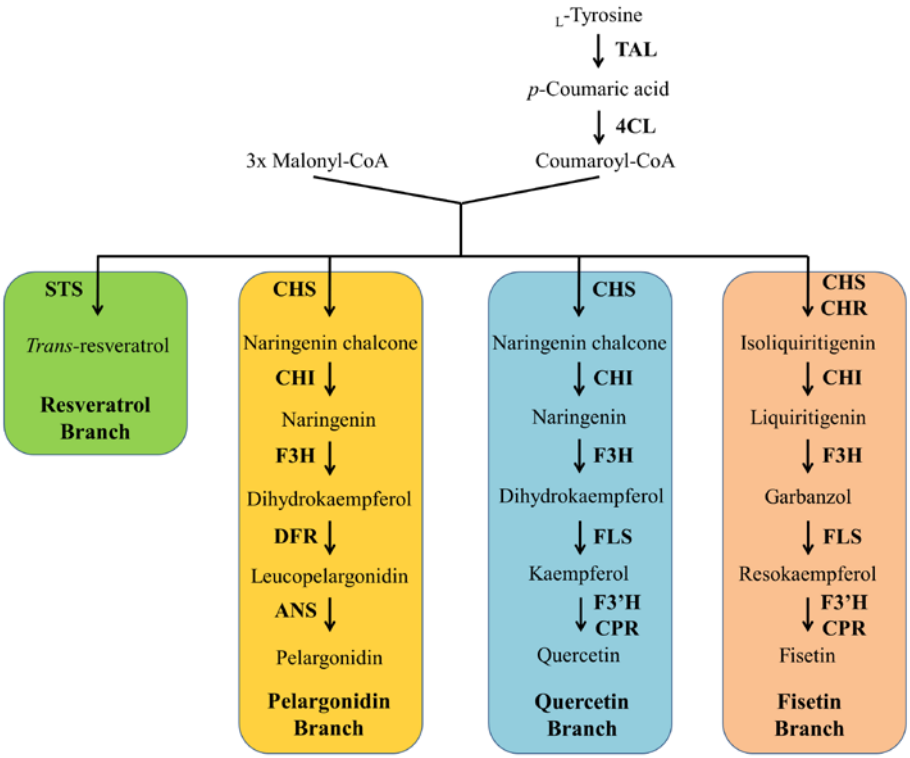

Figure 9: Heterologous biosynthetic pathways introduced into the genome-scale models of *C. glutamicum* and *L. lactis* leading to the four target compounds: i) *trans*-resveratrol ii) pelargonidin iii) quercetin and iv) fisetin. Two heterologous enzymes (TAL and 4CL) are common for the biosynthesis of the phenolic molecules. Enzyme abbreviations: TAL, tyrosine ammonia-lyase; 4CL, 4-coumaroyl-CoA ligase; STS, stilbene synthase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; F3H, flavanone 3–hydroxylase; FLS, flavonol synthase; F3'H, flavonoid 3' hydroxylase; CPR, cytochrome P450 reductase.

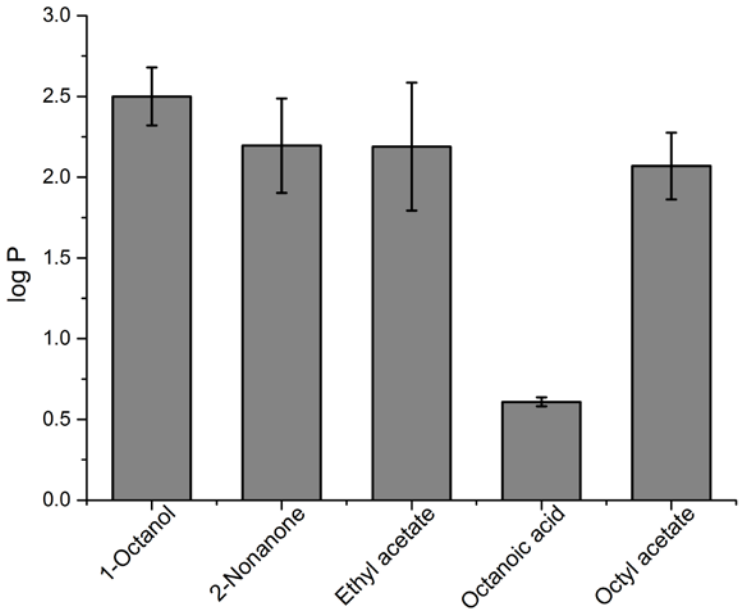

Figure 10: Partition coefficients in the form of log P values of *trans*-resveratrol using different organic solvents using *C. glutamicum* fermentation broth as the aqueous phase.

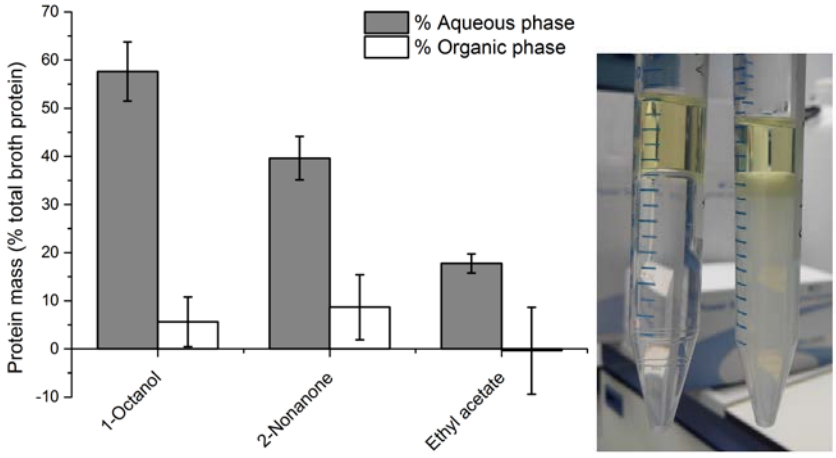

Figure 11: On the left, protein concentration (expressed as percentage of the initial broth protein concentration) in the organic and aqueous phases, after performing liquid extraction. The concentration in the organic phase is almost zero (considering the error bars), indicating that some protein stayed in the interphase. On the right, the formation of an interphase after the broth had been extracted with organic solvent (a mixture of heptanoic acid and hexyl acetate in this case) was clear.

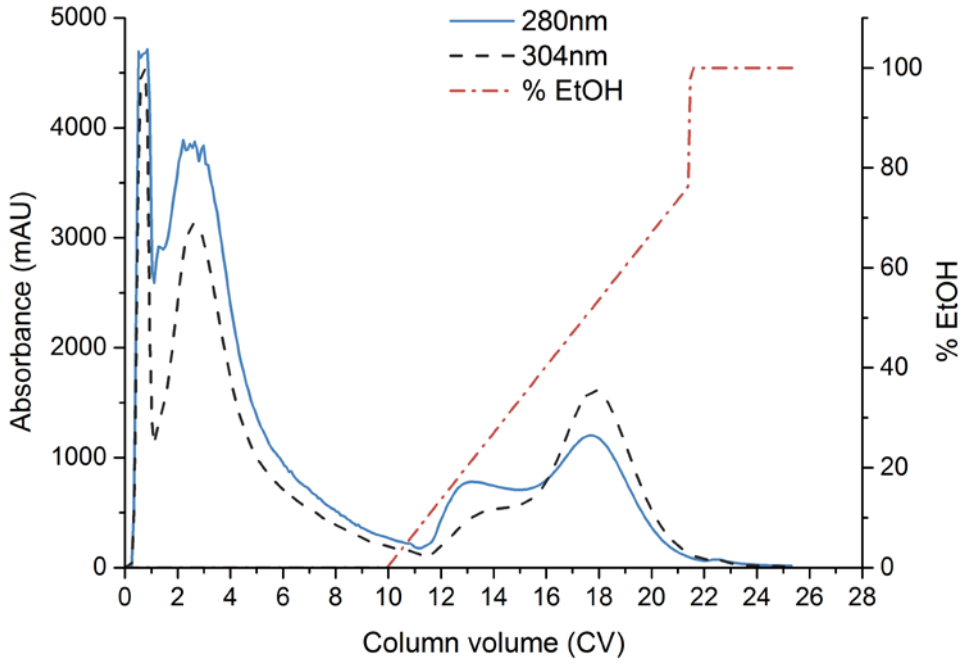

Figure 12: Chromatogram following a pulse injection of 10 ml of *C. glutamicum* spent fermentation broth in a 30 ml column packed with Amberlite XAD-16 resin. A washing step was performed with 10 column volumes (CVs) of MilliQ water. Afterwards, elution was performed with a water/ethanol gradient for 12 CV, followed by a final regeneration step with 100% ethanol.

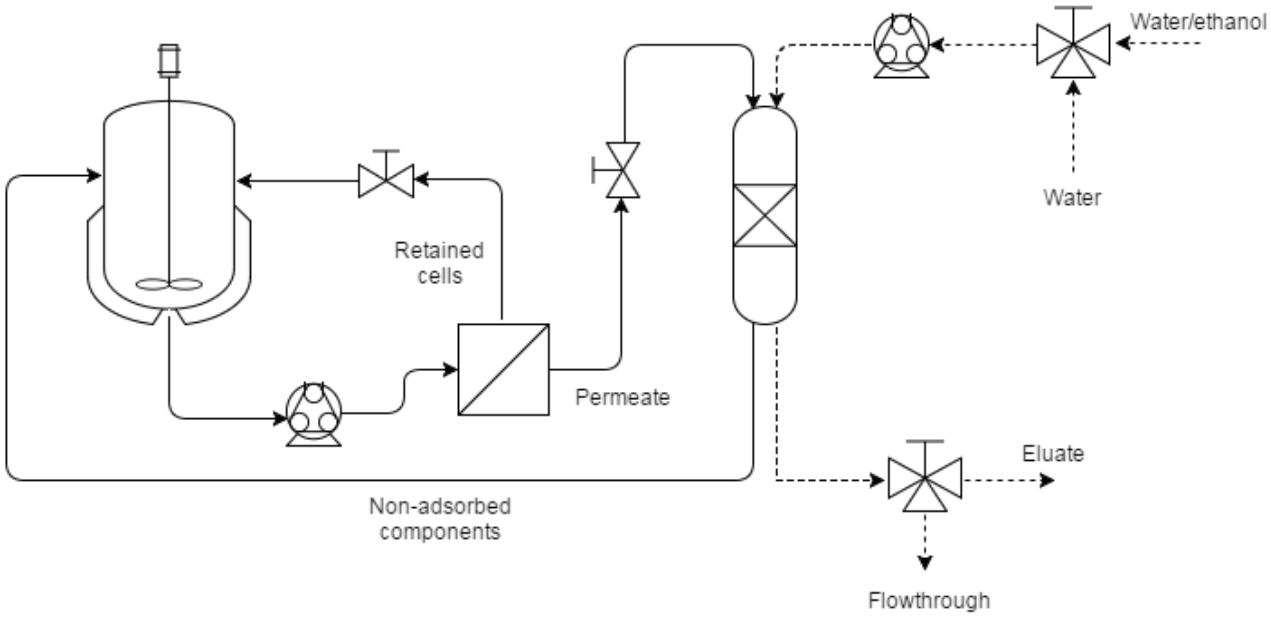

Figure 13: Experimental set-up for the “Continuous Product Removal” mode.

SUPPLEMENTARY TABLES

Title

BacHBerry: BACterial Hosts for production of Bioactive phenolics from bERRY fruits

Running Title

BacHBerry

Authors

BacHBerry consortium

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Table S1 – Decorating glycosyl groups of anthocyanins found in different berry species. The presence of a glycosylation pattern is noted with + whereas the absence is noted with -.

	3-O-arabinoside	3-O-glucoside	3-O-galactoside	3-O-rutinoside	3-O-sambubioside	3-O-sophoroside	3-O-xyloside	3,5-diglucoside	3-O-glucosyl-rutinoside	3-O-sambubiosyl- 5-O-glucoside	3-O-xylosyl-rutinoside	3-O-galactoside-5-O-glucoside	3-O-gentibioside	3-O-rutinoside-5-O-glucoside
Fragaria[1]	+	+	-	+	-	-	-	-	-	-	-	-	-	-
Ribes[1]	-	+	+	+	+	+	-	-	+	-	+	-	-	-
Rubus[1]	-	+	-	+	-	+	+	+	+	-	+	-	-	-
Vaccinium[1]	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Vitis[1]	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Lonicera[2]	-	+	-	+	-	-	-	+	-	-	-	-	+	-
Lycium[3]	-	-	-	-	-	-	-	+	-	-	-	+	-	+
Berberis[4,5]	-	+	-	+	+	-	-	+	-	+	-	-	-	+
Ugni[4]	-	+	-	-	-	-	-	-	-	-	-	-	-	-

[1] Phenol explorer database (http://phenol-explorer.eu)
[2] Chaovanalikit et al., 2004
[3] Zheng et al., 2011
[4] Ruiz et al., 2010
[5] Ruiz et al., 2013

Table S2 – Decorating acyl groups of anthocyanins found in in a variety of berry species

	3-O-(6'' acetyl-glycoside)	3-O-(6'' malonyl-glycoside)	3-O-(6''succinnyl-glycoside)	3-O-(6'' dioxyalyl-glycoside)	3-O-(6'' p-coumaryl-glycoside)	3-O-(feruloyl-glycoside)	3-O-(6'' caffeoyl-glycoside)
Fragaria[1]	-	+	+	-	-	-	-
Ribes[1]	-	-	-	-	+	-	+
Rubus[1]	-	+	-	+	-	-	-
Vaccinium[1]	+	-	-	-	-	-	-
Vitis[1,2]	+	-	-	-	+	-	+
Lycium[3]	-	-	-	-	+	+	+

[1] Phenol explorer database (http://phenol-explorer.eu)
[2] Figueiredo-Gonzalez et al. 2012
[3] Zheng et al. 2011

Table S3 – List of all the species/varieties and harvest locations of the germplasm collections

Species	Variety/line	Harvest location
Aristotelia chilensis (Molina)Stuntz	-	Chile[i]
Berberis buxfolia Lam.	-	Chile[ii]
Lonicera caerulea L.	260-11	Russia[iii]
Lonicera caerulea L.	838-12	Russia
Lonicera caerulea L.	Avacha	Russia[iii]
Lonicera caerulea L.	Bazhovskaya	Russia[iii]
Lonicera caerulea L.	Leningradskii Velikan	Russia[iii]
Lonicera caerulea L.	Malvina	Russia[iii]
Lonicera caerulea L.	Morena	Russia[iii]
Lonicera caerulea L.	Nimfa	Russia[iii]
Lonicera caerulea L.	Pavlovskaya	Russia[iii]
Lonicera caerulea L.	S322-23	Russia[iii]
Lonicera caerulea L.	Solovey	Russia[iii]
Lonicera caerulea L.	Start	Russia[iii]
Lonicera caerulea L.	Suvenir	Russia[iii]
Lycium chinense Mill.	-	China[iv]
Ribes spp. L.	1126	United Kingdom[v]
Ribes spp. L.	1196	United Kingdom[v]
Ribes spp. L.	2174	United Kingdom[v]
Ribes spp. L.	2292-1	United Kingdom[v]
Ribes spp. L.	644217	United Kingdom[v]
Ribes spp. L.	-	United Kingdom[v]
Ribes spp. L.	-	Chile[ii]
Ribes aureum Pursh	R2-1-2	United Kingdom[v]
Ribes bethmontii Jancz.	XW6	United Kingdom[v]
Ribes distans Jancz.	2269	United Kingdom[v]
Ribes divaricatum Dougl.	Montana	United Kingdom[v]
Ribes fuscescens Jancz.	XB6	United Kingdom[v]
Ribes futurum Jancz.	XR4	United Kingdom[v]
Ribes giraldii Jancz.	B2-3-1	United Kingdom[v]
Ribes grossularia L.	1815-21	United Kingdom[v]
Ribes grossularia L.	1824-37	United Kingdom[v]
Ribes grossularia L.	9492-5	United Kingdom[v]
Ribes grossularia L.	96101-1	United Kingdom[v]
Ribes grossularoides Maxim.	H1-12-1	United Kingdom[v]
Ribes holosericeum Otto& A.Dietr.	XR2	United Kingdom[v]
Ribes irriguum Douglas	H1-5-1	United Kingdom[v]
Ribes koehneanum Jancz.	XR3	United Kingdom[v]
Ribes nevadense Kellog	-	United Kingdom[v]
Ribes nigrum L.	Ben Alder	United Kingdom[v]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha*	Almiai	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Azhurnaya	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Bolero	Russia[iii]

Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum*	Buraya Dal'nevostochnaya	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Charovnitsa	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha*	Dochka	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Krasa L'vova	Russia[iii]
R.nigrum subsp. *scandinavicum x R. dikuscha*	Lazur'	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Malen'kii Prints	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Mila	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Monisto	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum x R.janczewskii*	Muravushka	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Ocharovanie	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Orlovskaya serenada	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Orlovskii Val's	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha*	Sensei	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Sharovidnaya	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha*	Syuita Kievskaya	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.nigrum* subsp. *scandinavicum*	Ukrainka	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Veloi	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Vospominanie	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha x R.nigrum* subsp. *scandinavicum*	Zelenaya Dymka	Russia[iii]
Ribes nigrum subsp. *europaeum x R. nigrum* subsp. *sibiricum x R.dikuscha*	Zhuravushka	Russia[iii]

Ribes petraeum Wulf.	R2-1-2	United Kingdom[v]
Ribes rotundifolium Michx.	H1-3-1	United Kingdom[v]
Ribes rubrum L.	Red Poll	United Kingdom[v]
Ribes rubrum L.	1518	United Kingdom[v]
Ribes rubrum L.	1169	United Kingdom[v]
Ribes rubrum L.	1196-2	United Kingdom[v]
Ribes rubrum L.	1757-2	United Kingdom[v]
Ribes rubrum L.	Red Wing	United Kingdom[v]
Ribes rubrum L.	S11-3-36	United Kingdom[v]
Ribes sativum Syme	R7-3-3	United Kingdom[v]
Ribes x *saundersii* Jancz.	XB4	United Kingdom[v]
Ribes succirubrum Zabel ex Jancz.	XW8	United Kingdom[v]
Ribes warcewiczii Jancz.	R7-3-3	United Kingdom[v]
Ribes watsonianum Koehne	-	United Kingdom[v]
Rubus armeniacus Focke [= *R. procerus* auct.])	Himalayan Giant	United Kingdom[v]
Rubus bartonii Newton syn. 'Ashton Cross'	Ashton Cross	United Kingdom[v]
Rubus fruticosus L. agg.	B13	United Kingdom[v]
Rubus fruticosus L. agg.	B14	United Kingdom[v]
Rubus fruticosus L. *agg.*	Fantasia	United Kingdom[v]
Rubus fruticosus L. agg.	Karaka Black	United Kingdom[v]
Rubus geoides Sm	-	Chile[ii]
Rubus ursinus x R. armeniacus x R. idaeus	Black Butte	United Kingdom[v]
R. ursinus x *R.ursinus* x *R.idaeus* x *R. ursinus* x *R. allegheniensis* x *R. argutus*	Kotata	United Kingdom[v]
Rubus ursinus x *R.ursinus* x *R.idaeus*	Ollalie	United Kingdom[v]
Rubus idaeus L.	Glen Ample	United Kingdom[v]
Rubus idaeus L.	Glen Fyne	United Kingdom[v]
Rubus idaeus L.	Malling Admiral	United Kingdom[v]
Rubus idaeus L.	Malmer Szedler	United Kingdom[v]
Rubus idaeus L.	Octavia	United Kingdom[v]
Rubus idaeus L.	Polana	United Kingdom[v]
Rubus idaeus L.	Prestige	United Kingdom[v]
Rubus idaeus L.	Tulameen	United Kingdom[v]
Rubus loganobaccus L.H.Bailey	Nectarberry	United Kingdom[v]
Rubus loganobaccus L.H.Bailey	Boysenberry	United Kingdom[v]
Rubus loganobaccus L.H.Bailey	Loganberry	United Kingdom[v]
Rubus loganobaccus L.H.Bailey	LY59-10	United Kingdom[v]
Rubus loganobaccus L.H.Bailey	Riwaka Choice	United Kingdom[v]
Rubus loganobaccus L.H.Bailey	Sunberry	United Kingdom[v]
Rubus loganobaccus L.H.Bailey	Tayberry	United Kingdom[v]
Rubus occidentalis L.	Black Hawk	United Kingdom[v]
Rubus occidentalis L.	Huron	United Kingdom[v]
Rubus occidentalis L.	Plum Farmer	United Kingdom[v]
Rubus phoenicolasius Maxim.	Wineberry	Portugal[vi]
Rubus brigantinus Samp.	-	Portugal[vi]
Rubus genevieri Boreau.	-	Portugal[vi]
Rubus henriquesii Samp.	-	Portugal[vi]
Rubus hochstetterorum Seub.	-	Portugal[vi]
Rubus sampaioanus Sudre ex Samp.	-	Portugal[vi]

Rubus vagabundus Samp.	-	Portugal[vi]
Ugni molinae Turcz.	Red Pearl	Chile[vii]
Vaccinium angustifolium Ait. ex Benth	Blomidon	China[viii]
Vaccinium corymbosum L.	Bluecrop	China[ix]
Vaccinium darrowii Camp	Misty	China[ix]
Vaccinium uliginosum L.	-	China[viii]
Vaccinium virgatum Ait.	Climax	China[ix]
Vaccinium vitis-idaea L.	-	China[viii]

[i] Sampling location: Coyhaique, Región de Aysén, Chile
[ii] Sampling location: Punta Arenas, Región de Magallanes, Chile
[iii] Vavilov Institute collection: Pavlovsk, St-Petersburg, Russia
[iv] Sampling location: Beijing botanical garden, Beijing, China
[v] The James Hutton Institute collection: Invergowrie, Scotland, UK
[vi] Propagation site: Fataca Experimental Field, Odemira, Portugal
[vii] Sampling location:Mataquito, Región del Maule, Chile
[viii] Sampling location: Tahe, Heilongjiang Province, China
[ix] Sampling location: Dalian, Liaoning Province, China

SUPPLEMENTARY MATERIALS

Title

BacHBerry: BACterial Hosts for production of Bioactive phenolics from bERRY fruits

Running Title

BacHBerry

Authors

BacHBerry consortium

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S1: Materials and Methods

Plant material and extraction procedure: A total of 112 different cultivars and species from the *Aristotelia*, *Berberis*, *Lonicera*, *Lycium*, *Ribes*, *Rubus*, *Ugni* and *Vaccinium* genera grown in different locations (see Supplementary Table 1) were harvested manually in the field and kept under cool conditions until they were transferred to -20ºC storage. Frozen fruit of each species/cultivar was weighed (approximately 50 g) into a solvent-proof blender containing 150 ml of pre-cooled 50ng ml^{-1} Morin (Sigma-Aldrich, Gillingham, UK) solution prepared with 0.2% formic acid in methanol solution. Samples were then homogenized and subsequently filtered using Whatman filter paper grade 1. The resulting filtrate was aliquoted and solvent-dried using a speed-vac followed by lyophilization. Dried extracts were flushed with N_2 and stored at -20ºC until analysis by LC-ToF-MS.

Phenolic profile determination by LC-ToF-MS: Dried extracts from each species/cultivar were resolubilized in triplicate using 2 ml of a 75% methanol solution with 0.1% formic acid. From these extracts, 500 µl were decanted into filter vials, sealed with 0.45 m PTFE-lined screwcap (Thomson Instrument Company, London, UK) and transferred into the autosampler. The analysis was achieved in positive and negative modes across 9 batches each, using an Agilent LC-ToF-MS system (Agilent Technologies, Cheadle, Cheshire, UK) consisting of a quaternary pump (Agilent 1260), a diode-array-detector (DAD) (Agilent 1260) a temperature control device (Agilent 1260) and a Thermostat (Agilent 1290) coupled to an Agilent 6224 time-of-flight (ToF) instrument. Five microliters of the sample were injected onto a 2x150 mm (4 µm) C18 column fitted with a C18 4 x 2 mm Security Guard™ cartridge (Phenomenex, Torrance, CA, USA). Sample and column temperatures were maintained at 4ºC and 30ºC, respectively. The samples were eluted at a flow rate of 0.3 ml min^{-1} using two mobile phases (A: 0.1% Formic acid in dH_2O; B: 0.1% formic acid in 50:50 dH_2O: Acetonitrile) with the following gradient: 0 min 5%B; 4 min 5% B; 32.00 min 100%B; 34.00 min 100% B; 36.00 min5% B; 40.00 min 5% B. For optimal electrospray ionization conditions the nebulizer pressure, drying gas temperature and drying gas were set to 45 psi, 350ºC and 3 l min^{-1}, respectively. In addition the diode array detection (DAD) was performed at 254, 280 and 520 nm. Morin levels (internal standard) were integrated in Agilent Mass Hunter Quan software (v. B.06.00) and all the samples with deviations larger than 10% relative to the dataset mean were reinjected. For all samples, three aliquots were analysed across three different analytical batches.

Component detection, peak alignment and integration: All chromatograms were processed identically using the Agilent Software Profinder v B.06.00 (Agilent Technologies, Cheadle, Cheshire, UK) which combines peak finding and integration algorithms for high-throughput data generation. For positive and negative mode data, the batch recursive molecular feature was used with peak extraction restricted to 2.1-38.00 mins of the chromatography and a peak threshold set at 15000 counts. The algorithm was set-up to include potential adducts of +H, $+Na^+$, $+K^+$ and $+NH4^+$ in positive mode and -

H and $+Cl^-$ in negative mode restricting the compounds to a maximum of one charge state. The compound ion count threshold was set at two or more ions, and for alignment purposes the RT window was set at 0.70% + 0.60 mins and the mass window was set at 25 ppm + 2 mDa. A post-processing filter to restrict analysis to compounds with more than 15000 counts and present in at least 3 of the files in at least one sample group (species/line). The find-by-ion options were set to limit the extracted ion chromatogram (EIC) to the expected retention time +/- 0.40 minutes. The "Agile" algorithm was used for the integration of EIC, with a gaussian smoothing of 9 points applied before the integration with a Gaussian width of 3 points. Additionally, peak filters were set at over 15000 counts and the chromatogram formats were set to centroid when available and otherwise profile. Spectrum was extracted at 10% of peak height and excluded if the spectra within the m/z range used was above 20% of saturation. Finally a post-processing filter was applied and compounds with less than 15000 counts or present in less 3 files in at least one sample group (species/line) were excluded.

Statistical analyses: Statistical analyses of all data were performed using GenStat for Windows, 16th Edition (VSN international Ltd., Hemel Hempstead, UK). A principal component analysis (PCA), based on the correlation matrix was applied to all the QC samples to ensure that the blank, reference samples, and berry samples were well separated (data not shown). Two separate analysis of the metabolomics dataset on the berry samples were carried out for the positive and negative dataset. A PCA, using the correlation matrix, was used to generate PCA plots of the first 4 principal components, which were subsequently used for selecting the species samples providing the most separation between samples.

Yeast plasmids, strains and transformation: p426_GAL1pr-GFP-Aβ42 was generated by inserting the sequence GFP-Aβ42, obtained by the double digestion of p416_GPDpr-GFP-Aβ42 (kindly provided by Ian Macraedie, RMIT University, AU) with *Bam*HI/*Sma*I, into the p426 vector. p425_GAL1pr-GFP-Aβ42 was generated by subcloning the sequence GAL1pr-GFP-Aβ42 into the *Sac*I/*Hin*dIII restriction sites of p425 vector. *S. cerevisiae* strains used in this study are BY4742 *erg6 MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ YBR082c::kanMX4* (EUROSCARF) and BY4742_*CDRE-lacZ MATa his3 leu2 lys2 ura3 aur1::AUR1-C-4xCDRE-lacZ* (Prescott et al, 2010).

Yeast growth conditions and extract treatments: Synthetic complete (SC) medium [0.67% (w/v) yeast nitrogen base without amino acids (YNB) (Difco) and 0.79 g l^{-1} complete supplement mixture (CSM) (QBiogene)], containing 2% (w/v) glucose was used for growth of Crz1-reporter strain. Crz1 activation was induced with 1.8 mM $MnCl_2$ (Stathopoulos & Cyert). FK506 (Cayman Chemicals) was used as positive control. Synthetic dropout (SD)-LEU-URA media [0.67% (w/v) YNB and 0.54 g l^{-1} 6-amino acid dropout $CSM_{-ADE\text{-}HIS\text{-}LEU\text{-}LYS\text{-}TRP\text{-}URA}$ (QBiogene)], supplemented with standard concentrations of the required amino acids and containing 1% (w/v) raffinose, were used for growth

of the AD yeast model. The repression or induction of disease protein expression was carried out in medium containing glucose (control, disease-protein OFF) and galactose (disease-protein ON) at a final concentration of 2% (w/v).

A pre-inoculum was prepared in appropriated raffinose or glucose (only for Crz1-activation model) medium and cultures were incubated overnight at 30°C under orbital shaking. Cells were diluted in fresh medium and cultures were incubated under the same conditions until the optical density at 600 nm ($OD_{600\ nm}$) reached 0.5 ± 0.05 (log growth phase). To ensure synchronized cells cultures, cell suspensions were further diluted according to the equation ODi x Vi = (ODf/($2^{(t/gt)}$) x Vf, where ODi = initial optical density of the culture, Vi = initial volume of culture, ODf = final optical density of the culture, t = time (usually 16 h), gt = generation time of the strain and Vf = final volume of culture. Readings were performed in a 96-well microtiter plate using a Biotek Power Wave XS plate spectrophotometer.

Growth assays: Yeast cultures were diluted to $OD_{600\ nm}$ 0.12 ± 0.012 in fresh medium supplemented or not with the indicated concentrations of extracts in a 96-well microtiter plate. After 2 h incubation at 30ºC, cultures were further diluted to $OD_{600\ nm}$ 0.03 ± 0.003 in medium containing glucose or galactose and supplemented or not with extracts. The cultures were then incubated at 30°C with shaking for 24 h or 48 h (for the AD model) and cellular growth was kinetically monitored hourly by measuring $OD_{600\ nm}$. The areas under the curve (AUC) were integrated using the Origin software (OriginLab, Northampton, MA).

β–Galactosidase assays: For quantitative measurements of *β*–galactosidase activity, $OD_{600\ nm}$ of cells cultures were recorded just before cell lysis. Cells were then incubated with Y-PER Yeast Protein Extraction Reagent (ThermoFisher Scientific) in 96-well microtiter plates for 20 min at 37ºC, after which LacZ buffer containing 4 mg l^{-1} ONPG was added and plates incubated at 30ºC. The $OD_{420\ nm}$ and $OD_{550\ nm}$ was periodically monitored using a Biotek Power Wave XS Microplate Spectrophotometer until the development of the yellow color. Miller units were calculated as described previously (Garcia et al, 2016).

Fractionation of bioactive fruit extracts: The freeze-dried extract of selected berries (eg *Rubus genevieri*, and *Rubus idaeus*) were re-suspended in Milli-Q water, filtered and adsorbed onto a semi-preparation column packed with a C18 resin (PREP C18 55-105 μm 125Å, Waters Co. USA) using an AKTA Explorer (GE USA). 0.1% Formic Acid (Sigma, USA) in Milli-Q water was chosen as mobile phase A and Methanol (Chromasolv purity≥99.9%, Sigma, USA) as mobile phase B. The approach was modified for the different berry extracts (*Rubus genevieri*, *Rubus idaeus*) to achieve proper fractionation, by tuning the hydrophobicity of the mobile phase (water-methanol). Modifying loading

and elution mobile phase composition and gradient profile served to generate a maximum number of well identifiable peak/fractions for further bio-activity testing (ranging from 12 to 28 fractions). Fractions were brought under vacuum using a rapid-vac (Labconco, USA) to remove methanol for further bioactivity testing.

References

Garcia G, Santos CN do, Menezes R (2016) High-Throughput Yeast-Based Reporter Assay to Identify Compounds with Anti-inflammatory Potential. In: Matthiesen R (ed) Proteostasis. Springer, New York, USA, pp 441–452

Prescott TAK, Ariño J, Kite GC, Simmonds MSJ (2012) Inhibition of human calcineurin and yeast calcineurin-dependent gene expression by *Jasminum humile* leaf and root extracts. J Ethnopharmacol 140:293–297. doi: 10.1016/j.jep.2012.01.020